BIOLUMINESCENT ACTIVITY IN THE MATING AND ANTIPREDATORY BEHAVIOR OF A MARINE OSTRACOD (CRUSTACEA, MYODOCOPIDA)

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About an hour after the sun sets a complex and ritualized light show of precise, vertically placed luminescent pulses erupts over the shallow grassbeds of the western Caribbean throughout the year. These are the most complex displays known in marine systems. Displays consist of repeated trains of secreted bioluminescent pulses in a specific pattern ejected into the water column for courtship by male *Vargula annecohenae*, small (<2mm) myodocopid ostracod crustaceans. Quantification via the use of image intensification and infrared videography shows that each 40-cm long luminescent display train consists of a **stationary phase** of 3 (usually) brighter, longer pulses placed close together, followed by a **helical phase** of about a dozen evenly placed dimmer, shorter pulses secreted by an individual male rapidly spiraling upward. The operational sex ratio in the display grounds above the grassbeds is highly skewed toward males (>175:1). Each participating male is capable of 1) **initiating** a luminescent display train, 2) **entraining** on another displaying male in loose luminescent synchrony, and 3) **'sneaking'** silently on a luminescing male, and can switch among these three tactics during a single train. Which alternative mating tactic is chosen is predicted by the orientation and distance of the responding male from the initial courtship display at the start of that tactic. Unlike fireflies, females do not respond with luminescent signals of their own. Instead, by using a light-emitting-diode array to mimic a male display, I show that females respond to and intercept the

intermittent luminescent displays by compensating their trajectories between each light pulse. Besides courtship, all individuals of *V. annecohenae* (males, females, and juveniles) respond to predation attempts by their nocturnal predators by releasing enormous quantities of luminescence. Since individuals are distasteful to their predators, the signals probably function as aposematic signals, but also as lures for predators of an attacker (burglar alarm). Based on photomultiplier tube recordings of 1) courtship displays, 2) antipredation displays, and 3) total luminescent available, a male could produce nearly 500 courtship trains or 4 major antipredation displays from its existing stores.

BIOGRAPHICAL SKETCH

Trevor Rivers was born in Fayetteville, North Carolina on July 14, 1978. Soon after he moved to Waterville, Washington, where he spent his formative years riding his bicycle, watching marine biology programs on PBS, and digging in his back yard. In the fourth grade, one assignment was to write a science paper, and his teacher Mrs. Bradley gave him the topic of bioluminescence. It was a hard topic, especially as there are no fireflies on the West Coast for examples, and even harder for Trevor's mother, Kathi, to help find articles that would make even a little bit of sense to a fourth-grade kid. Ever since then, Trevor has had a fascination with luminescence, and wrote research papers on it in high school, community college at Wenatchee Valley College, and as an undergraduate at Western Washington University (WWU).

At WWU, Trevor majored in marine biology and was lucky enough to participate in the NSF-funded Science Education And Research for UNdergraduates (SEARUN) program in 1998, where he helped study the effects of temperature and UV-B radiation on the health of corals in the Bahamas. This whetted his appetite for marine research, and the next summer (1999), he interned at the Monterey Bay Aquarium Research Institute (MBARI), where he worked with Drs. Steve Haddock and Bruce Robison on trying to discover why a population of *Aequorea victoria* jellyfish cultured in the Monterey Bay Aquarium lost the ability to luminesce. They found that these jellyfish needed to obtain certain chemicals in their diet, and Trevor decided that luminescence research was definitely where his interests belonged.

After graduating in 2000 from WWU, Trevor moved to Oakland, California, where he worked as a program analyst for the US Coast Guard Icebreaker Operations. He helped primarily with the science support of the Arctic missions and spent some time up above the Arctic Circle on the vessel The Polar Star. It was there, in subzero temperatures, 60-foot seas, and 55-degree rolls, that he decided that tropical research

was probably the way to go for graduate school. He met his advisor Jim Morin at the International Society for Bioluminescence and Chemiluminescence meeting in Asilomar, CA, in 2000, was accepted to the Department of Ecology and Evolutionary Biology for 2001, and wrote this dissertation in 2007.

I dedicate this dissertation is dedicated to my grandfather, Donald 'Doc' Rivers, the man who most inspired me to become a scientist. As a high school math and science teacher with two master's degrees (as well as someone who had stints as an ambulance driver and city policeman, among other things) he was someone whose interests paralleled my own. I had long wished for him to be there when I received my diploma, but he passed away in June 2003 when I was in the middle of my field season in Belize. "Grandpa, there's a 'Doc' Rivers in the family again."

ACKNOWLEDGMENTS

There are many people that I would like to thank for various reasons. I would like to thank my fourth-grade teacher, Mrs. Bradley for introducing me to the topic of bioluminescence, Rob Fitch at Wenatchee Valley College for fueling my interest in marine biology, and Dr. Gisele Muller-Parker at Western Washington University for being a great advisor and giving me the opportunity to do real research as an undergraduate. Drs. Steve Haddock and Bruce Robison at the Monterey Bay Aquarium Research Institute were key in my realizing my dream of doing luminescence research in the marine environment. I would like to thank my graduate advisor and committee chair Dr. Jim Morin for a great study system and great insight.

I would like to thank Jennifer Hall, John MacDougal, and others at International Zoological Expeditions (IZE) on Southwater Caye, Belize, for help and accommodations during our field research. I would also like to thank committee members Drs. Tom Seeley and Paul Sherman for their reviews, Gretchen Gerrish for research help, among many other things, and Francoise Vermeylen and Myra Shulman for statistical support. Roberto Torralbas, an undergraduate at Cornell, was critical in the design of our computer-controlled display mimic. I'd also like to thank all of the other graduate students in EEB (to whom I will claim to be environmentally conscientious by not wasting more paper and ink by naming each one here), in particular my long-time housemate and friend Dan Lebbin, for both academic and non-academic support. I grateful to DeeDee Albertsman, Carol Damm, Linda Harrington, Rosie Brainard, Alberta Jackson, Patty Jordan, LuAnne Kenjerska, Brian Mlodzinski, Janeen Orr, and Patty Jordan, the administrative staff in EEB for helping keep academic life more organized than it otherwise would have been. Dr. Kevin McGraw at Arizona State University was kind enough to let me work in his lab for a few months while I finished writing this dissertation.

I’m grateful to the Department of Ecology and Evolutionary Biology for summer research support, Cornell University for a one-year fellowship, and research grants from the Cornell chapter of Sigma Xi and the Andrew K. Mellon foundation. I’d also like to thank the Orenstein fund and Cornell University for help in defraying costs of conference travel.

Finally, I’d like to thank my entire family, including my parents Greg and Kathi Shannon, my aunt and uncle Marcia and Rob Smith, and grandmother Nadra Rivers for moral, financial, and overall support. And a special heartfelt thanks to Yexin Jessica Li, for always being there in every possible way, and making life and graduate school a much, much brighter place.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

IR	Infra-Red
LED	Light-emitting diode
NVD	Night-vision device
PMT	Photomultiplier tube

CHAPTER 1

A DESCRIPTION OF THE COMPLEX COURTSHIP DISPLAY OF *VARGULA ANNECOHENAE,* A MARINE BIOLUMINESCENT OSTRACOD

Introduction

Where mate choice is exhibited by one or both sexes, a signal must be received by the discriminators. Signals can be vocal, olfactory, tactile, or visual, and can range from simple, such as a pheromone plume, to highly complex, as found in the visual and auditory displays of some birds (Andersson, 1994). In order to infer which aspects of a signal are important, signal characteristics must be quantified.

Visually-based mating systems are more common in diurnal than nocturnal animals. However, some nocturnal organisms utilize visual displays involving luminescent signals, such as fireflies. Firefly studies include descriptions of the luminescent display and patterns by displaying males (Lloyd, 1966), reveal which aspects of the displays are attractive to females (e.g. Branham and Greenfield, 1996; Lewis and Wang, 1991; Michaelidis et al., 2006; Vencl and Carlson, 1998), and discuss male-male interactions (Buck 1988; Copeland and Moiseff, 1997a, 1997b). Other firefly studies describe mimicry and tracking by extra-specific females for predation purposes (Lloyd, 1975, 1980; Lloyd and Wing 1983).

Although the number of luminescent marine species exceeds luminescent terrestrial species (Hastings and Morin 1991), luminescent sexual displays in marine environments are not well known, largely because of the difficulty of *in situ* observations. Research on syllid polychaetes ("fire-worms") reveal that a female, either floating at the surface or rising from the benthos will glow for many seconds or minutes to attract conspecific males, which intermittently and rapidly flash when approaching (Markert et. al., 1961; Tsuji and Hill, 1983; Morin, pers. com.). The luminescent courtship displays of male cypridinid ostracods (Myodocopida, Ostracoda,

Crustacea) in the Caribbean have proven to be a much more complex, more akin to firefly displays than "fire-worm" displays (Herring, 2000; Morin, 1986; Morin and Cohen, 1991). Over a dozen species of ostracods can be found in specific habitat types (gorgonian patches, coral types, sand patches, grassbeds etc.) within a single Caribbean reef system. Each species has a dramatically different light display. Each display train is secreted as multiple, multi-compound chemical packets into the water column (Morin, 1986; Morin and Cohen, 1991). The luminescent compounds are synthesized in a luminescent organ made up of long secretory or exocrine cells, with each one traveling the entire length of the light organ and terminating at nozzles on the upper lip (Huvard, 1993). Muscle bands around and through the light organ apparently contract, squeezing the compounds into the water in species-specific patterns (Huvard 1993). Depending on the species, males can display while swimming upwards, downwards, diagonally, or horizontally, with bright blue pulses that vary from about 100ms to >10s depending on the species. Of the more than 60 known species of displaying ostracods in the Caribbean, there is no known case of a luminescent 'duet' or 'dialogue' between males and females. Females do not produce light during courtship bouts, although they luminesce when attacked by a predator (Morin, 1986; Morin and Cohen, 1991). The lack of dialogue between the sexes, the complexity and diversity of the signals among species, and the fact that the luminescence is an extracellular secretion mark these cypridinid systems apart from other luminescent courtship systems currently known. Morin (1986) tentatively classified the mating system a spree, or temporal lek (*sensu* Walker, 1983), with individuals only entering the water column (lek area) for courtship during a specific twilight time window. Fertilization is internal, females brood young internally, and males give no parental care (Cohen and Morin 1990; Gerrish et al. submitted), but there is evidence of female choice (Rivers Chapter 3).

Previous research on luminescent ostracods primarily addressed questions regarding their phylogeny, systematics, display patterns and distributional differences among species (Morin and Cohen, 1991; Cohen and Morin, 1990, 2003 and references therein). Because of the difficult nature of this system (i.e., working with small [ca. 2mm], fast-swimming [up to 15cm/s] marine crustacea that intermittently luminesce in the dark in the open sea), little is known beyond basic descriptions of the luminescent patterns in the field.

We discovered that infrared (IR) light reflects sufficiently off the carapaces of individual ostracods in clear acrylic sea water tanks, to enable the use of low-light CCD cameras to observe in detail ostracod behavior during courtship displays. Here, we provide a detailed description of the male mating display and the behavioral patterns of one signaling species, *Vargula annecohenae*.

Background of the life history patterns and luminescent displays of Vargula annecohenae

One of the most abundant western Caribbean luminescent ostracod species, *V. annecohenae* is commonly found in the shallow seagrass beds of Belize. This species is the only luminescent ostracod found in abundance in grassbeds in Belize and can be collected in great numbers using traps baited with fish muscle (we collect juveniles and adults, males and females). As with all other cypridinid ostracods, *V. annecohenae* has a life cycle that includes reproduction by copulation with internal fertilization, brooding by females, crawl-away juveniles (i.e. there is no planktonic larval stage), and 5 discrete juvenile instars that lead to a single terminal adult stage (Cohen, 1983; Cohen and Morin, 1990; Gerrish and Morin, *submitted*). There is clear sexual dimorphism, with females being much larger than males; males are 1.62 +/- 0.05 mm in length while females are 1.99 +/- 0.05 mm. The life span in the lab can be

up to 9 months and the time from brooded egg to adulthood is about 3 months (Gerrish and Morin, J. Crust. Biol. submitted)

The courtship displays of *V. annecohenae* are trains of vertically placed short pulses of light that are easily quantifiable in space and time. The display periods are synchronized with the lunar cycle, with the most activity occurring when the moon is not present or is low in the sky; there is no activity during the full moon (Gerrish *et al*., in press). At a precise "dark threshold," approximately one hour after sunset or moonset, whichever occurs later (Gerrish et al., in press), males participate in mating displays above the grassbeds of Belize for approximately an hour.

Males can exhibit one of several alternative mating tactics: 1) **initiate a display** on their own, 2) **entrain** (synchronize) their flashing pattern on that of an already displaying male, or 3) **sneak** silently above a luminescing male (Rivers, Chapter 3).

Each display train appears to have two distinct phases. The first, or ***stationary phase***, consists of 3-4 (variable) bright pulses with some interpulse interval variation, and occurs at or just above the top of the grass (ca. 15-20 cm above the substratum). These pulses show no distinct upward movement, although some lateral movement may occur. The second, more uniform (in space and time) portion, which we call the ***helical phase*** (see below for explanation), occurs as a series (10-15) of somewhat dimmer, upwardly-placed shorter pulses with more consistent interpulse intervals and interpulse distances. The total vertical length of a display train is about a maximum of 60 cm upward in the water column.

Methods

Field Experiments and Observations

Collection of ostracods: Ostracods for lab trials were collected off the southwest shore of Southwater Caye, Belize [16.801° N Lat, 88.083 ° West Long],

from 15 January to 10 February 2006. We used small (4 cm diameter x 8 cm long) PVC-pipe traps with a 500 μm mesh funnel at each end, and fish muscle as bait, similar to methods found in Cohen and Morin (1986). Males were maintained in seawater in 750 ml Gladware containers until used in a trial. All ostracods were returned to the grassbeds following the experiments.

Collection of ostracods during luminescent displays: A 500μ mesh cloth sweep net [25 cm diameter, 50 cm length] was used to collect ostracods during the displays. We waited underwater until a male started the helical phase of the display (usually the third pulse), thus minimizing collecting unwanted particulates such as grass blades and other organisms in the net, and then raised the net around the display from below and twisting closed the net after each sweep. We repeated this procedure throughout the display period. The netted males and females were placed in seawater in a bucket and taken to the lab where they were sorted, separated by sex, counted, and their average numbers per display calculated. Then they were stored in Gladware "aquaria." The ratio of males to females provided us with the operational sex ratio (OSR) from the proximity of the displays.

Male display density: A 0.25m^2 square quadrat [50cm on a side] made of 1.25 cm diameter PVC pipe was haphazardly placed on the grassbed in ca. 2m of water off the south beach of Southwater Caye, Belize. To accomplish this, one diver, either on snorkel or using scuba, rotated on the sea surface with eyes closed, tossed the quadrat and let it settle to the bottom, and then recorded how many displays (including displays that were entrained with earlier displays) were observed in the water column directly above the quadrat in 3 minutes. For non-random, high-density sampling, the quadrat was placed on the grassbed adjacent to a small (1m x ½ m) dead coral rubble head where we had observed consistently high numbers of displays over multiple nights. We again recorded the number of displays in the quadrat observed in 3 minutes. We counted 3 (2 random, 1 nonrandom) quadrats within the first 30 minutes after the

first displays started, and again after 60 minutes from the start of the first display. We used a log-transformed random effects mixed model (SAS 9.1) to compare the densities between random and nonrandom samples.

Number of pulses per display: In situ videos of the courtship displays were recorded using a Dark Invader Generation II night-vision device (NVD) attached to a Sony DCR VX-2000 camcorder, in a custom Aquavideo underwater video housing and positioned perpendicular to the displays and parallel to the sea floor. The numbers of pulses per display from individual displays were taken from the video files. We also performed field censuses by counting the pulses from individual displays, while we were either on snorkel or on scuba, and writing the results on an underwater slate.

Lab Experiments and Observations

Two-Dimensional Recordings: To control the start of displays in the lab, males were maintained in the Gladware "aquaria" under natural conditions from the night of their collection, and under a 15-watt fluorescent light until being used for their trial during the second night after collection. For each trial, at least 4 males were placed in a clear acrylic tank with dimensions of either 60cm (height) x 70cm (width) x 15cm (depth) (hereafter called the 'large' tank) or 60cm x 15cm x 16cm (hereafter called the 'small' tank) filled with clean seawater collected off the dock on the lagoon-side of Southwater Cay near the display grounds. We used a minimum of 4 males because we were usually unable to elicit displays consistently with fewer than 4. For each experimental trial, a 15-watt fluorescent light was left on above the tank for 20 minutes and then extinguished. We began recording when the displays commenced, usually within 10 to 45 minutes. If 45 minutes passed without displays, new males were substituted. Infrared illumination for filming was supplied by a rheostat-controlled 15-watt red frosted incandescent bulb further restricted by an infrared barrier filter situated 1cm above the waterline. The output from a high-sensitivity

(0.00015 lux) low light 1.25 cm CCD camera (Watec LCL-902K) with a 12mm aspherical low-light TV lens (Computar HG1208FCS-HSP) situated about 2 meters away and on the side of the tank was fed into a Sony DCR VX-2000 miniDV camcorder, which we used as a VCR. This system allowed us to follow most of the behavioral activity of each of the males in the tank during and between displays. Trials were recorded for either 30 or 60 minutes.

Three-Dimensional Recordings: To observe the display in three dimensions, two low light (0.00015 lux) CCD cameras (Watec LCL-902K) with low-light aspherical lenses were used to film the top and bottom of the front of the tank, while a third, more distant CCD camera similarly equipped filmed the side of the tank (Figure 1). In addition, a Dark Invader Generation II NVD equipped with a 3-mm BG-39 barrier filter (to block out IR light) fed into a Sony DCR VX-2000 camcorder and also recorded the same field as the side camera. All four images were connected to a 30 frames per sec (fps) black and white digital quad-processor. This arrangement allowed for all 4 cameras to be displayed on one screen, a Canon ZR 85 Mini-DV camcorder was used as a VCR. Two of the CCD cameras were closer in front (1 m), and one CCD camera was aligned in tandem with the NVD system farther away (2 m) on the side (**Figure 1.1)**. The IR light was placed as in the 2-D arrangement. With this method we could follow the individual activities of each male with the IR light (CCD cameras) and the luminescent displays [NVD-equipped camera] simultaneously in three dimensions.

Photomultiplier tube (PMT) setup and analysis: For all experiments involving the use of light-intensity recording, we used a horizontally-placed RCA 931-A photomultiplier tube (PMT), covered by an Andover 039FG11-50 3mm Infrared (IR) barrier filter, at a distance of 76 cm from the experimental tank. The PMT was powered by an Emco Ca12N High voltage converter (set to 1000v). The PMT output was connected to a Dataq DI-158U analog data acquisition device and set to a gain of

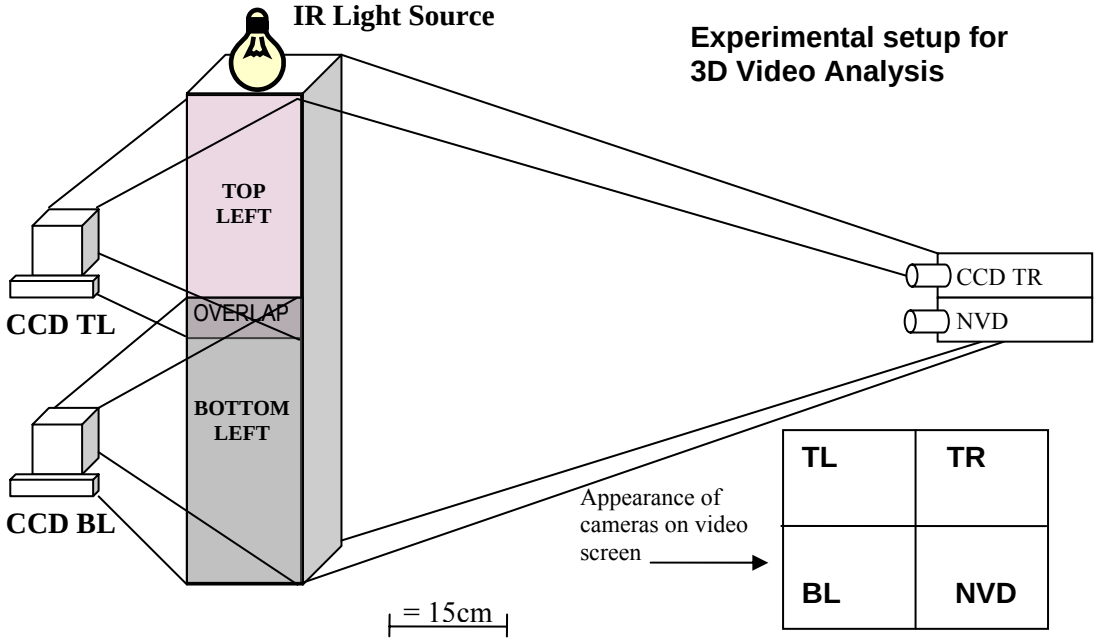


Figure 1.1 Three-dimensional recording method. For close viewing, two low-light CCD cameras split (with overlap) the front of the tank (TL = top left, BL = bottom left of the image panel), and one CCD camera (TR = top right of the image panel) and a video camera with night-vision device (NVD) in tandem recorded movement and luminescence, respectively, from the side. The image panel box at the lower right indicates the image partitioning of the recordings.

8. Data were recorded at a rate of 240 data points per second on a Dell laptop, using the waveform analysis program WinDAQ. Using this program we were able to determine relative intensity, pulse duration, and interpulse intervals of the displays.

Data Analysis of the Luminescent Displays:

Maximum luminescent intensities: The maximum pulse intensities of 78 displays over five trials were used to determine variations within and among displays. We calculated the maximum variation in light intensity that could be attributed to location in the tank and distance from the PMT by using the inverse square law (intensity at a given distance = source intensity/4πdistance^2) for distances between 78-82.4 cm (minimum to maximum) from a display in the tank to the PMT, and the attenuation of light passing through seawater (the maximum distance of 21.5cm x the coefficient of 0.00015 yields an attenuation value of 0.003). Subtracting this value from the variation found in intensities of the displays gave us the variation in displays due to luminescence output among displaying males.

Interpulse distances and intervals: For interpulse intervals, our aim was 1) to determine the differences between the stationary and helical phases of individual display trains and 2) to characterize the differences in intervals within the helical phase.

We calculated interpulse intervals using two separate methods. First, to obtain the most accurate intervals, we used the waveform data of 20 representative display trains (longer, uninterrupted, and with clean peaks for analysis) from the PMT data (at a resolution of 240 data points per second) to find the interval from the beginning of one pulse to the beginning of the next. Because variations were relatively low, these results were used to produce a model for a typical display. Second, to determine amount of variation between individual males, we analyzed 2-dimensional videos of male behavior of 85 displays in 5 trials in the small tank. Since we had only video

images and not waveform data that came from known different individuals, the resolution was restricted to the speed of our DV camera (single frame = $1/30^{th}$ second = 33 ms). The projected image of the male's location was marked on a projection board, digitized, and analyzed with Image-J software. Because the stationary phase is more variable than the helical phase and the helical phase is evident by a distinct change in pattern, starting the analysis with the helical phase rather than the first pulse of the stationary phase provided a more accurate estimate of the variations. We used a random effects mixed model (SAS) to correct for multiple observations of the same male within treatments.

For interpulse distances, we analyzed 2-dimensional vertical and horizontal distances between pulses on the projection board. The scatterplot of our data showed a parabolic trend, so a quadratic equation in a random effects mixed model analysis was used.

After obtaining the mean interpulse intervals and distances from laboratory trials, we used them to extrapolate the display duration and length of field displays with the mean and maximum number of pulses per display. The reason extrapolation is necessary is that because in the field, with a moving camera, multiple nearby displays, and a large depth-of-field, accurate determinations of interpulse intervals and distances are difficult. We counted the number of pulses per display in the field of 23 displays by eye while snorkeling, then used the mean intervals and durations between each pulse to calculate the mean and maximum display lengths and durations.

Three-Dimensional swimming patterns and speeds: In the small tank, the helical portions of 8 males' display trains and the entire display trains of 4 additional males were analyzed in 3-dimensions in order to determine the pattern of swimming of both the stationary and helical phases, and to compare to the 2-dimensional helical calculations of swimming speed during the helical phase. The 2 front cameras and 1 side camera were size-standardized in Image-J, and the male's position (in 3D space)

was marked every 2 frames (1/15th second =67 ms). Cartesian coordinates in three planes were plotted and point-to-point distances and speeds were subsequently calculated. Due to the nature of the film and tanks, in order to observe the stationary phase we had to choose only those males (N= 4) that started their displays high enough off the bottom and far enough from the sides of the tank to prevent potential edge effects. Since these males were higher in the tank, their displays only consisted of 9-10 pulses before reaching the surface, rather than the 15-19 possible from males starting their displays at the bottom of the tank. A paired t-test was used to compare actual 3-dimensional swimming speeds with 2-dimensional transformed data.

Results

Observations and recordings of the luminescent courtship displays of the cypridinid ostracod *Vargula annecohenae* show consistent patterns with respect to habitat and display period in the field, and display train characteristics and pulse characteristics in the laboratory **(Tables 1.1 - 1.3, Figure 1.2)**. A single display, which consists of a series of discrete extracellular pulses of light-producing products, shows two major phases: 1) an initial stationary phase composed of 3-4 brighter, closely placed and slightly longer pulses, followed by 2) a more conserved and less variable helical phase composed of many regularly spaced in both space and time, dimmer and shorter pulses of similar intensity and duration to one another and laid down by a spirally swimming male **(Figures 1.2,1.3)**. Our definition of 'stationary' does not mean the male itself is stationary, but that the males are looping back to nearly the same location and secreting the following pulse close to the previous *Display period and density*: In the field, the display arena occurs in the water column immediately above the seagrass bed. Displays start from 0 to 10 cm above the tops of the *Thallasia* seagrasses and, extrapolating from laboratory interpulse distance data, proceed upward for a mean distance of 30.5 cm, with a maximum of 60 cm. Displays commence

Table 1.1 General Display Characteristics of *Vargula annecohenae* in Belize

		Mean	SE	Max	N
Display habitat	Shallow grassbed				
Start time of displays after sunset	45 +/- xx min		?	?	?
Absolute time (min)*					
Relative time (crep units)*			?	?	?
Display direction	Vertically Upward				
Start distance of display above top of seagrass (cm)	0-10 cm				
Inter-display intervals from end of one display to start of the next (s) **		19.15	2.09	67.5	85
Mean number of males per display – field data		2.47	0.83		868
Mean number of females per display – field data		0.014	0.005		868
Male/Female operational sex ratio per display	176:1				

* Data from Gerrish et al., pers. com

** Lab IR data with 5 males per trial

Table 1.2. Display train characteristics

Total Train:	Mean	SE	Max	N
Field Observations and Extrapolations				
Total pulses per train	12.26	0.911	19	23
Train length (vertical)[= helical phase] (cm) $^{\Delta}$	38.19		64.72	
Train duration (s) $^{\Delta}$	10.10		14.12	
Lab Observations and Extrapolations				
Total pulses per train *	12.79	0.30	19	161
Train length (vertical)[= helical phase] (cm) – lab (extrapolated to 13 pulses, limited by tank)	27.91	0.93	41.2	85
Train duration [time until displaying male dropped] (s)-lab (limited by tank)**	10.86	0.29	16.3	85
Overall 3D swimming speed (cm/s)	7.76	0.55		4
Stationary Phase:				
Swimming speed (cm/s)	7.16	0.137	9.2	4
Distance: horizontal (cm)	-0.08	0.240	5.26	85
Distance: vertical (cm)	-0.31	0.21	3.59	85
Helical Phase:				
Pulse number after which helical phase starts	2.85	0.02	6	85
Swimming speed (cm/s)	8.39	0.192	13.3	8
Vertical swimming speed (cm/s)†	5.41			
Mean width of helix (cm)	0.73	0.02	0.83	8

$^{\Delta}$ Distances and durations extrapolated from filed pulse number and lab IP Intervals and distances

* Lab NVD data with 5 males per trial

**Lab IR data with 5 males per trial

† Calculated from mean IP distances and elapsed times of 85 displays

Table 1.3. Characteristics of pulses in the luminescent courtship displays of the cypridinid ostracod *Vargula annecohenae* based on laboratory recordings of the **stationary** (gray) and **helical** (white) phase (mean +/- SE [n]). * = no data available.

Pulse Number	Intensity (as % of Pulse 1)	Pulse Duration (ms)	Interpulse Interval (s)	Interpulse Distance (Vertical)(cm)	Interpulse Distance (Horizontal)(cm)
1	100	430+/-20 [16]			
2	57.3 +/- 5.5 [19]	330+/-18 [16]	1.30+/-0.05 [85]	-0.07+/-0.24 [85]	-0.28+/-0.21 [85]
3	35.34 +/-3.4 [19]	260+/-10 [16]	0.98+/-0.04 [85]	1.55+/-0.17 [85]	0.32+/-0.15 [85]
4	30.31 +/-4.4 [19]	260+/-10 [16]	0.83+/-0.01 [85]	2.53+/-0.17 [85]	-0.10+/-0.16 [85]
5	23.77 +/- 4.7[19]	240+/-9 [16]	0.76+/-0.01 [83]	3.41+/-0.15 [83]	0.21+/-0.19 [83]
6	20.35 +/- 4.3[18]	220+/-10 [16]	0.73+/-0.01 [81]	3.81+/-0.15 [81]	0.24+/-0.24 [81]
7	15.68 +/- 2.7[17]	210+/-6 [16]	0.73+/-0.01 [76]	4.05+/-0.15 [76]	0.38+/-0.33 [76]
8	15.32 +/-3.2 [17]	210+/-10 [15]	0.69+/-0.01 [69]	3.92+/-0.18 [69]	-0.13+/-0.07 [69]
9	14.53 +/- 1.3[17]	190+/-7 [15]	0.71+/-0.01 [51]	4.04+/-0.19 [51]	0.45+/-0.46 [51]
10	14.13 +/-3.4 [14]	180+/-8 [15]	0.69+/-0.03 [43]	3.73+/-0.19 [43]	0.00+/-0.07 [43]
11	12.30 +/- 2.5[12]	190+/-9 [13]	0.67+/-0.01 [21]	3.57+/-0.23 [21]	-0.26+/-0.08 [21]
12	13.75 +/- 3.2[9]	180+/-11 [8]	*	*	*
Mean Stationary		340+/-13 [48]	1.23 +/-0.04 [160]	-0.31+/-0.21 [82]	-0.08+/-0.23 [82]
Mean Helical	18.41+/- 1.5[122]	210+/-3 [129]	0.75+/-0.007[494]	3.77+/-0.05 [494]	0.23+/-0.10 [494]

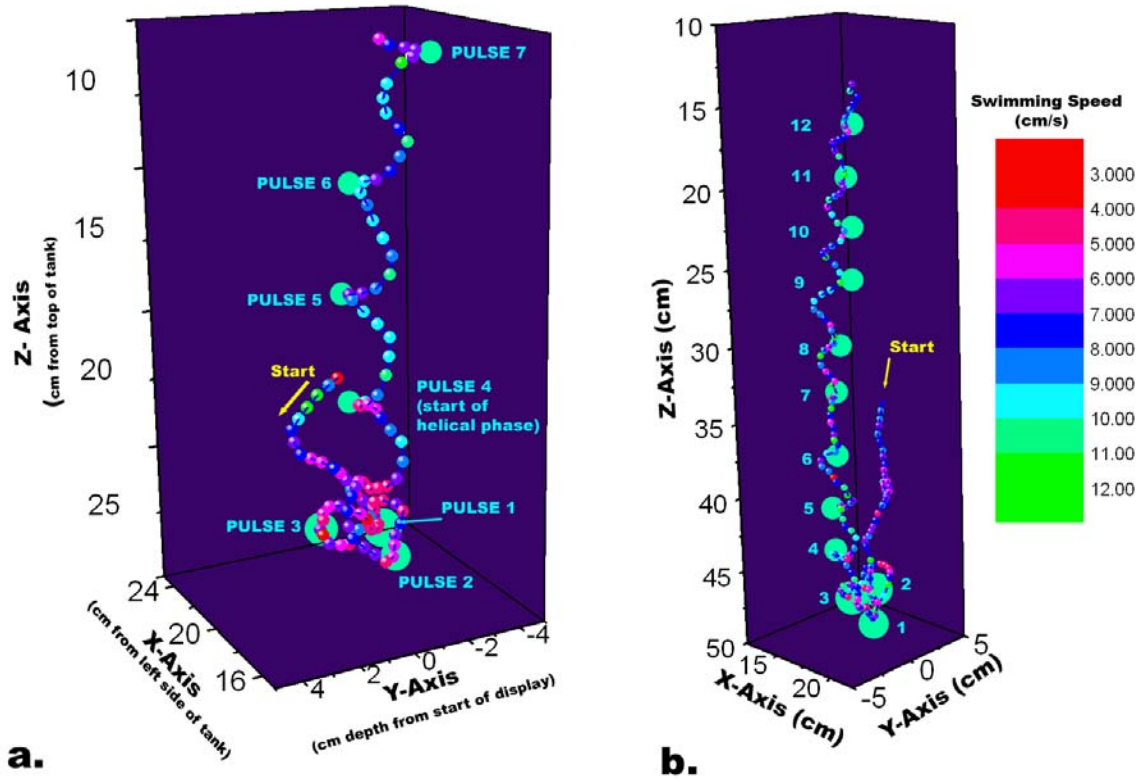


Figure 1.2. Three-dimensional examples of two male ostracod display swimming patterns and speeds. The Z-axis corresponds to cm from the top of the tank, the x-axis is the distance from the left side of the tank, and the Y-axis is the distance in depth from the first data point. Data points are every 67 milliseconds. **a. Close-up of the first 7 pulses of a display**. The color of the sphere corresponds to the swimming speed of the ostracod at that given point in time. Males swimming in the stationary phase swim slower than when in the helical phase (see also Figs. 3d & 4). **b. Example of an entire display**. The male in this instance released its first pulse nearly 20 cm from the bottom of the tank, then continued swimming straight toward the bottom of the tank before luminescing again.

toward the end of twilight (45 minutes after sunset) and last for about 60 min (**Table 1.1)**, with an abrupt initial increase and later a gradual decrease in display density over this period. These luminescent courtship displays occur abundantly over the shallow grassbeds at Southwater Cay, Belize, and, based on random samples, average about seven displays per square meter per minute duringpeak activity **(Table 1.1)**. Specifically, based on 33 random counts, we found 1.78 (SD 1.28, SE 0.23) displays per ¼ m^2 quadrate in the more homogeneous grassbed area. Where unattached, but at least temporarily stable, coral heads were situated within this homogenous environment, we documented 14.37 (SD 8.80, SE 2.35 [N=13]) per ¼ m^2, or nearly 60 displays per square meter per minute in these hotspot areas, which is significantly higher than the homogeneous grassbed numbers (DF 31, $f = 25.41$, $p < 0.0001$ between the log-transformed data from randomly-thrown quadrats and the known high display-density area near the coral head). However, this species does not display in areas far from seagrasses, such as sand areas, cobble or coral, all of which have their own habitat-specific displaying species.

While the overall population has an even male-female sex ratio (Gerrish and Morin, submitted), luminescent courtship display arenas are highly male-biased. We collected about two and a half males from each display, but only slightly more than one female in every one hundred displays (sweeps from 868 displays yielded a mean number of 2.47 males and 0.014 females per display)**(Table 1.1)**. This difference gives a female: male operational sex ratio (OSR) of 0.00567, or 176 males per female **(Table 1.1)**.

Display characteristics: Each train is quite predictable, uniform and repetitive. It is composed of an initial ***stationary phase*** followed by a rapid upward production of slightly dimmer, more regular pulses in a ***helical phase***. In the field, there was a mean of 12.26 (+/- 0.91 SE, N = 23) total pulses per display, with a maximum of 19 pulses

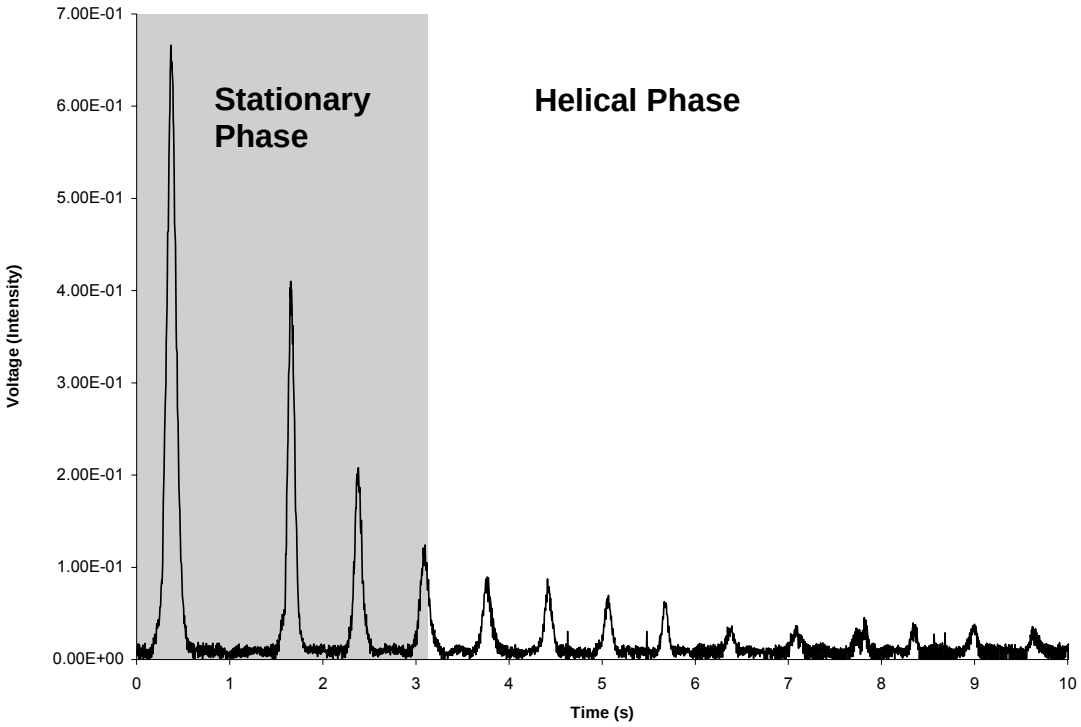


Figure 1.3. Typical photometric waveforms from a single display. The first 3-4 bright pulses are typical of the stationary phase and the helical phase of the remainder. The mean duration of each pulse also decreases during the stationary phase, but becomes more consistent during the helical phase (see also Fig. 1.4b). Light intensity is directly correlated to the voltage output of the photomultiplier.

(**Table 1.1)**. Using the mean interpulse interval durations collected in the lab (since these data are more accurate than field data as discussed above), the mean duration of each display train (from the beginning of the first pulse to the end of the last pulse) was calculated to be 10.1 s in the field **(Table 1.2).** Because intensities, pulse duration, interpulse intervals and interpulse distances plateau by about pulse 12, we used the helical pulse lab data to project the characteristics of the remaining 7 helical pulses to obtain the maximum distance and duration of displays in the field. Although the spatial structure of displays are quite uniform, displays vary considerably in their brightness. Based on controlled laboratory observations, across 78 displays, the brightest first pulse was 84 (+/-4) % brighter than the dimmest first pulse. Using the dimensions and distances of the photomultiplier from our observation tank and the inverse square law, we calculated the maximum variation due to variation in distances from the display to the PMT to be 15%. Therefore, for each case, at least 69% (+/- 4%) of the variation of signal intensities during a trial can be directly attributed to the variation in actual display luminescence intensities. Since we were unable to match displays to individual males, we do not know whether this variation is only between males, or may even occur within a single male.

Based on an analysis of the displays, there was no detectable horizontal component to the train, but there was a distinct vertical pattern with each display terminating at a maximum of about 60 cm above the top of the grassbed. There were significant differences between both interval number ($f = 165.23$, $p<0.0001$) (Table 2, Figure 4d) and the square of the interval number ($f = 102.00$, $p <0.0001$), which means that interpulse vertical distance increased then decreased following the quadratic equation:

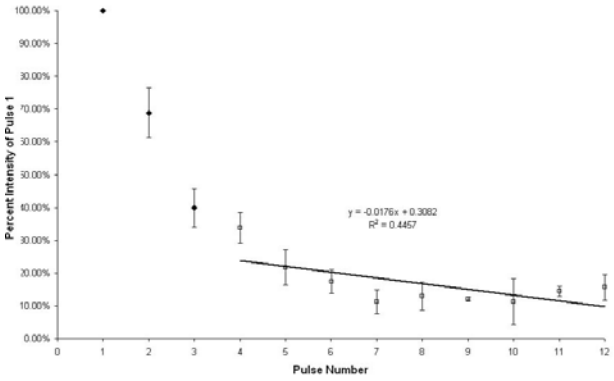
$$\text{Distance} = 1.9385 + 0.8379(\text{IP Interval number}) - 0.07067(\text{IP Interval number})^2$$

We also found that the duration of interpulse intervals was found to differ significantly between males (DF= 101, f = 2.13, p =0.0095).

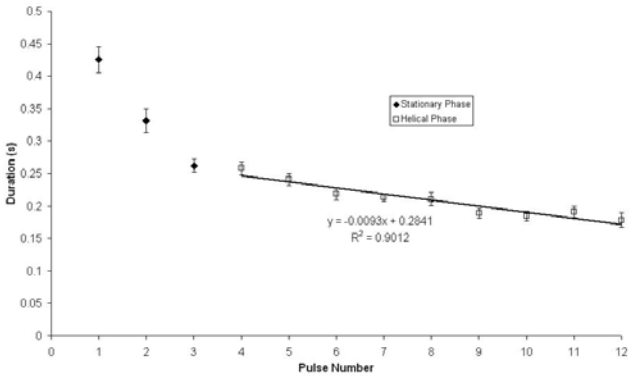
Stationary phase: The stationary phase of the display does not demonstrate any distinct spatial pattern other than issuing a luminescent "call" followed by the male looping up and back down a short distance and then luminescing again, often in nearly the same location or slightly laterally (**Figure 1.2)**. During the (usually) three pulses of this phase intensities, durations and interpulse intervals all decline from one pulse to the next **(Table 1.3)**. The mean intensity of luminescent pulses decreased (**Figure 1.3, Figure 1.4a),** and dropped to below 40% of the first pulse intensity by the third pulse **(Table 1.3, Figure 1.4a).** The mean pulse duration also decreased during the stationary phase from about two-fifths of a second to a quarter of a second **(Table 1.3, Figure 1.4b)**, and interpulse intervals decreased by about half from more than a second to about 650ms **(Table 1.3, Figure 1.4c).** There was no trend for any vertical or horizontal movement during the stationary phase **(Table 1.3, Figure 1.4d)**. The pulse intensity during the helical phase decreased with pulse number, as in the stationary phase, but only from about 24% of the first pulse to about 14% at the 12^{th} pulse (with some of this variation possibly due to distance of the pulse to the PMT) **(Figure 1.4a, Table 1.3)**. The mean pulse durations decreased from 260 ms to 180 ms duration, with a mean decrease of 9.5 ms per pulse (r^2 =0.89). The mean interpulse intervals stayed consistent at approximately 0.6s **(Table 1.3, Figure 1.4c)**, showing no trend to decrease as interval increased (r^2 = 0.29). Similarly, once a plateau was reached at about pulse five, the interpulse distance was also nearly constant at slightly less than 4 cm (Table 2, Figure 4d). The vertical (apparent) speed of a display production during the helical phase remained fairly constant, at 5.41 cm/s **(Table 1.2, Figure 1.5)**, but the mean 3-dimensional helical phase (actual) speed, i.e. the male swimming in a tight upward spiral, was 8.39 (+/- 0.19) cm/s **(Table 1.2)** with the

Figure 1.4. Mean values of a typical male display. Bars are Standard Error; refer to table 2 for n-values. **a. Percent intensity of the 1st pulse.** Helical phase pulse intensities are approximately 10% of the initial stationary pulse [N=16]. **b. Mean pulse duration:** The mean pulse duration decreases rapidly during the stationary phase, then only slowly during the helical phase [N =16]. **c. Mean interpulse interval**. The mean interpulse intervals vary considerably in the stationary phase, but are highly consistent during the helical phase [N=85 pulse 1, see Table 1.2 for remainder]. **d. Mean distances traveled.** There is no trend for horizontal movement during the course of a display, but vertical distance increases during the stationary phase, then levels off during the helical phase [N=85 pulse 1, see Table 1.2 for remainder].

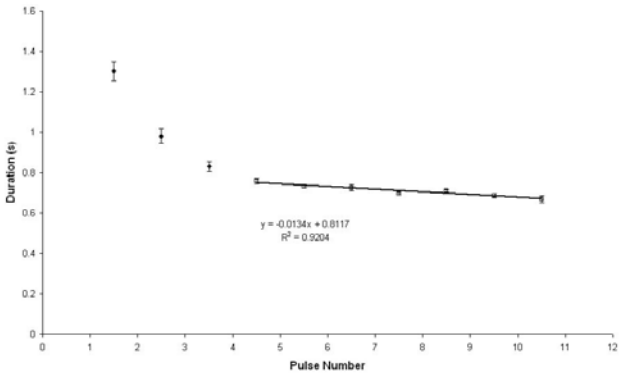
a. Mean Percent of the Intensity of the 1st Pulse



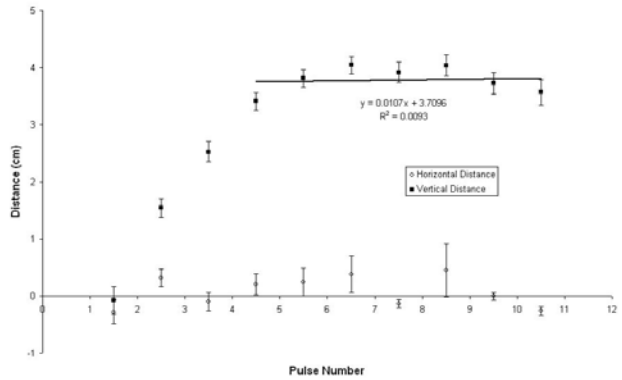
b. Mean pulse duration



c. Mean interpulse interval



d. Mean horizontal and vertical interpulse distances



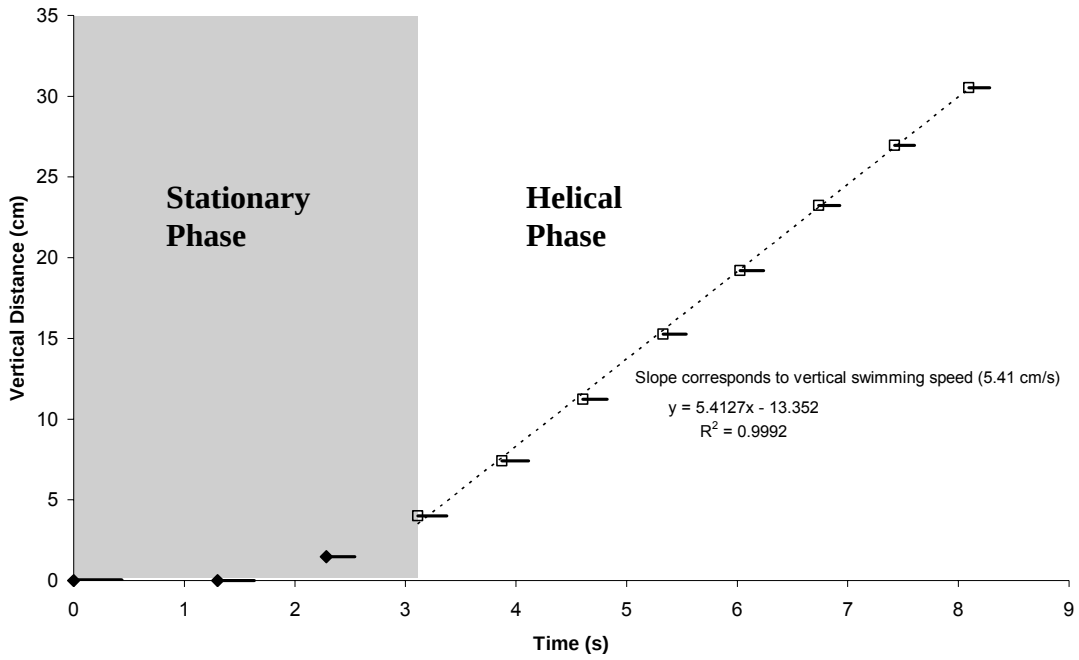


Figure 1.5. Vertical position of ostracod per time using the mean IP intervals and IP distances from video analysis, and pulse durations from photomultiplier recordings (from Table 2). Data points correspond to vertical distance traveled after each flash. Closed diamonds correspond to the stationary phase and open squares to the helical phase of the display. Horizontal solid lines correspond to pulse duration. The vertical swimming speed of 5.41 cm/s, calculated from the regression line (dotted line) during the helical phase, is consistent over the course of a display. As we used the mean time and distances (Table 1), error bars are not appropriate.

width of the helical cylinder being about 7.3 mm. Since there was no significant difference between 3-dimensional actual display speeds and a helical swimming speed calculated from 2- dimensional analyses ($t = -0.98553$, $p = 0.35719$), the 2-dimensional calculations are representative to true mean swimming speed during the helical phase of the display and can be used for most analyses from a single-angle camera. Observations of the 3-dimensional swimming pattern of males during the helical portion of the display suggest that males are swimming in both right-handed and left-handed helices, but whether individuals always exhibit the same handedness in their spirals is unknown.

Discussion

On first observation of the high-density displays in the field, it is difficult to detect how displays are dispersed throughout the grassbed habitat. Although grassbeds are for the most part homogenous in their composition, we found that there are three separate display density phenomena. There are 1) lower-density display areas that cover huge swaths of seagrass beds, 2) predictable hotspots, and 3) some ephemeral hotspots. The predictable and ephemeral hotspots may be formed for entirely different reasons. Predictable hotspots are occasionally found adjacent to semi-stable intrusive reef materials (e.g. a dead coral head), which tend to collect high levels of biological activity. The ostracods (both male and female) could be drawn to these sites as food-rich areas, or they could provide access to their (as of yet unknown) diurnal resting place, which may increase the probability of encountering females. This high display activity could form as predicted by the 'hotspot' model which states that display arenas are chosen for reasons such as being on or near female feeding grounds (Bradbury and Gibson 1983, Bradbury et al. 1986). On the other hand, the formation of ephemeral hotspots may be due to the attractiveness of certain signalers to not only females, but competing males. Since multiple males respond to a single display in the

surrounding area (Rivers, Chapter 2), this clumping could then further induce a cascade of clustering of male displays in the homogenous grassbed areas. Therefore, the formation of ephemeral hotspots may be more in line with the 'hotshot' hypothesis, where males cluster around displaying 'hotshot' males (Beehler and Foster 1988). Regardless of what causes the clustering of male displays in both predictable and ephemeral hotspot areas, the high display numbers may attract females at a higher rate, thus allowing them more opportunities for choice in a small area. Although a hotspot area may increase the number of females that may potentially respond to a signal, there is also a concomitant increase in competing males. If there is a large variation in male fitness in the population (which is likely given the skewed OSR), with displaying males tending to have higher reproductive fitness than sneakers, there may be a potential downside in hotspot activity in that a female may be more likely to be intercepted by sneaking males than in more homogeneous, lower-density situations.

These ostracod luminescent display patterns are unique among courtship displays in a number of ways. First, the luminescent courtship displays are extracellular and secreted into the water column by rapidly swimming males (over 40 body lengths per second on average). Because of this speed, by the time the luminescence has reached its peak intensity (100-150ms), the male is already about 5-10 mm away from the pulse. Further, since there is no luminescent dialogue between males and females (unlike fireflies) where males and females might orient to each other using reciprocated signals, there is the question of how a female can get close enough to a chosen male to mate. Our evidence suggests that females, and also competing males, approach a displaying male silently (i.e. without luminescing) and stereotypically for interception (Rivers, Chapters 2, 3). We hypothesize that the two dramatically different but predictable phases of the display, the stationary and helical

phases, impart different information to both responsive females and 'eavesdropping' males.

Since the swimming pattern is not predictable, the stationary phase appears to be an attention-grabbing signal, and imparts little information for orientation for observers. For the purposes of this hypothesis we refer to this stationary phase in functional terms as an alerting and assessment (or call) phase because it appears to alter the behavior of both receptive females and competing males (Rivers, Chapters 2, 3). It notifies conspecifics that a new display is about to commence. This phase takes place at or just above the tips of the seagrass blades, and the pulses are longer-lasting and up to 85% brighter than those in the later helical phase **(Figure 1.4a, Table 1.3)**. Furthermore, there is variation in pulse number and interpulse intervals, so that during this phase it is difficult to predict precisely where along the top of the grassbed the displaying male is located immediately after a pulse. This lack of horizontal reference does not allow for precise localization of the signaler, but it does both alert attracted parties to the presence of a pending display and the general vicinity of the event. The observations that females require at least two luminescent pulses before responding to a display (Rivers, Chapter 3) and that males need at least 2-3 pulses before starting an alternative mating tactic (Rivers, Chapter 2) lend further support that this phase is an alerting signal. General bioluminescence in grassbed areas is also not restricted solely to ostracod mating displays; there may be potentially other luminescent signals from dinoflagellates, syllid polychaetes, occasionally displays from other ostracod species, or from predation attempts on ostracods (pers. obs.). Responding erroneously to these spurious luminescent signals could be prevented by requiring at least 2-3 pulses before males and females commit to a response.

The helical phase of the display, however, is highly consistent in all aspects of the display and provides a stereotypical pattern that conspecifics can approach. Thus,

for our communication hypothesis we consider this helical phase in functional terms to be the orientation phase, wherein approaching conspecifics can use the predictive aspects of the signals to accurately approach and intercept a displaying male. The display arena is the water column above the grass beds, which means that responding individuals (both male and female) could be anywhere in three-dimensional space, although females are likely to be above the displays (Rivers Chapter 3). Therefore, it is more crucial during this phase for the male to behave in a predictable way to be able to maximize its likelihood for successful interception by, and copulation with, a receptive female. Females approach luminescent signals by swimming in a trajectory to intercept the male above the most recent pulse, which is where he will be within a fairly narrow spatio-temporal range, thus supporting this hypothesis (Rivers Chapter 3). In addition, the majority of responding males that perform alternative mating tactics (entraining or sneaking) begin *after* the helical phase starts, indicating the shift to the helical phase is important for competing males as well (Rivers, Chapters 2).

Using lab observations of interpulse interval durations, distances, 2-and 3-dimensional swimming patterns, and intensities, we have been able to construct a model of the luminescent courtship display behavior of an average male *Vargula annecohenae* **(Figure 1.6)** that very closely resembles actual 3-dimensional display and swimming patterns (**Figure 1.2).** When a male begins displaying, he either drops to or loops at the level of the seagrass, releasing a bright pulse of luminescence. This is followed by 2-3 more pulses yielding the attention-grabbing ***stationary phase***. The male then swims vertically in a tight helical pattern (***the helical phase)*** with predictable distances and intervals between pulses. If a display is successful in attracting a female it ends. Termination of an unsuccessful display appears to occur when a male either reaches the sea surface or reaches the maximum number of pulses per train (19); interference from other males also may cause display termination. Then

the male swims directly down, without a spiral, to the seagrass and often starts again. We have documented repeated displays multiple times in the lab, and are confident that males behave similarly in nature.

Before a male's display can be used for courtship, it first must be recognized as a signal from a conspecific and not a spurious signal from another ostracod species or other luminescent organism. Pattern recognition of visual and auditory signals by members of the same species has been extensively studied amongst many organisms (crickets, frogs, fish, birds, etc.), with call frequency, intervals, and pattern providing important cues (Becker, 1982; Doherty and Hoy, 1985; Michaud, 1962; Morris and Fullard, 1983). The courtship displays most akin to ostracod displays are produced by fireflies, and we hypothesize that similar means of coding species identity are used in both of them. The interpulse intervals of multiple species of *Photinus* fireflies have been found to be integral to female response to luminescent cues; if the intervals are outside a critical range (either too long or too short), there is no female response (Lloyd, 1966; Michaelidis et. al, 2006), which may imply females are not recognizing such a signal as a courtship signal. *Photinus* fireflies respond to flashes in laboratory settings to a stationary flash, without needing other spatial cues such as distances traveled between flashes, etc. for pattern recognition. We hypothesize, however, that the spatial patterns in ostracods will prove to be as important as the timing for species and mate recognition. Ultimately, by modifying displays in laboratory settings using LED lights, we should be able to determine the thresholds at which *V. annecohenae* no longer recognize a display to be emitted by a conspecific.

Once a signal has been recognized as a conspecific mating display, various aspects of the signal should impart information regarding the quality of the displayer, which could then be used for female choice (see Andersson 1994 for review). The probability of female choice in the *V. annecohenae* mating system is quite high as

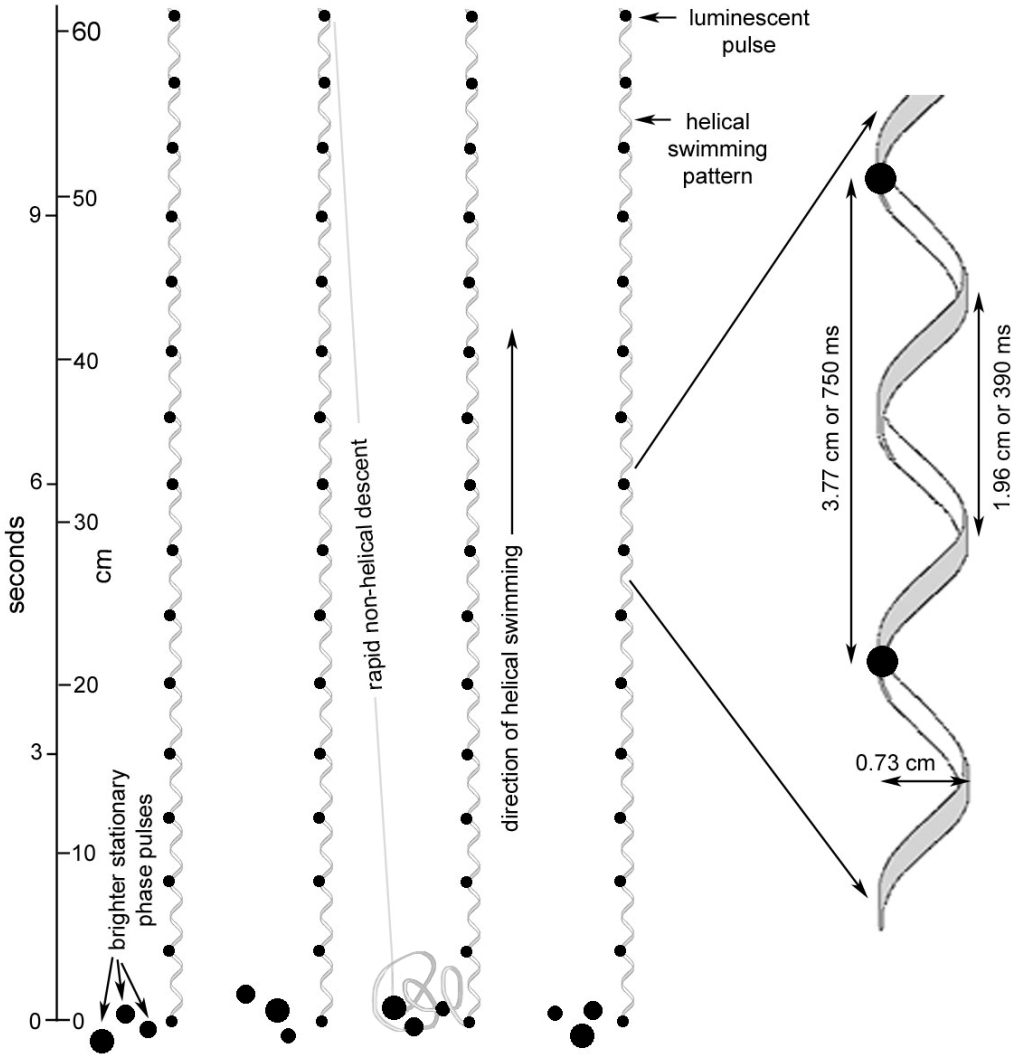


Figure 1.6. Model of a single male displaying 4 successive times. Using the interpulse intervals, interpulse distances, pulse intensities, and swimming patterns of males displaying in the lab, we show a typical male displaying 4 times. Black dots are the location of the pulses, and their size corresponds to relative intensity. The first three pulses are in the stationary phase, the remainder in the helical phase. The third display train shows a typical stationary phase swimming pattern. A male reaches the top, drops straight down, and then commences displaying again.

suggested by the skewed operational sex ratio (>176:1 male: female), the ability of females to avoid unwanted copulation, and the female behavior of tracking and intercepting light displays (Rivers, Chapter 4). However, for female choice to occur there must be some variation among displaying males (Shuster and Wade, 2003). Indeed there are significant differences among individual males in terms of interpulse intervals during the helical phase (Rivers, Chapter 2).

The intensity of a display (visual, auditory, or chemical) has been hypothesized to be a character on which females exhibit choice, and has been found to be important in a wide variety of organisms (Arak, 1983; Bailey et. al, 1990; Moore, 1988; see Andersson, 1994 for review), including fireflies (Cratsley and Lewis, 2003; Vencl and Carlson, 1998). The more variation between displays, the more selective a receptive female can be between potential mates. We observed a wide variety (with some displays over 70% brighter than others) of luminescent intensities in *Vargula annecohenae*, and although we were not able to simultaneously track individuals and record luminescence intensities, we hypothesize that further studies that follow individuals throughout a display period will show significant intensity differences among them. If intensity is the best indicator of male quality, one would expect a female ostracod to change her trajectory towards the brightest pulse that she can recognize as a conspecific courtship signal.

Although interpulse intervals and interpulse distances may be important for species recognition and orientation, and there is evidence that female *Photinus consimilis* fireflies prefer faster flash rates (which corresponds to shorter interpulse intervals) (Branham and Greenfield, 1996), female *V. annecohenae* may not be choosing between males by these signal features. We hypothesize that males whose displays most closely match the approaching swimming trajectories of approaching

females will be the most successful regardless of intensity, simply by increasing the probability of interception. For example, a male swimming too slowly may cause a female to swim too far above the display, causing her to be potentially intercepted by the sneaker males that primarily swim above the displaying male (Rivers, Chapter 2). Swimming too quickly, on the other hand, could cause the male to pass above a responding female without detecting her. Since the vertical speed of a displaying male is faster than the swimming speed of a female, the female would not be able to overtake a displaying male if below a displaying male and must necessarily be above the display (Rivers, Chapters 2 and 4). Thus, female response behavior may be causing stabilizing selection on the interpulse intervals and distances; if the intervals are either too fast or too slow, or the distances too long or too short, the male will be unsuccessful in finding and mating with a female. If there is stabilizing selection acting on interpulse intervals and distances, the intensity of the signal may prove to be the most important aspect of the display for female choice.

Conclusion: The luminescent displays of Caribbean ostracods are the most complex found in the marine environment to date, and rival or even exceed those of terrestrial fireflies. The grassbed species *Vargula annecohenae* is found in prodigious quantities and produces huge numbers of displays nearly every night of the year throughout the grassbed habitats of Belize and probably beyond (Gerrish et al. in press). The complexity of this system extends well beyond the luminescent displays, however. The extremely skewed nearly 176:1 male : female sex ratio in the water column is indicative of high levels of male competition as well as the potential for significant female choice. Males switch between entraining their own luminescent signal with that of a displaying male, or sneaking silently on displaying males with a rapidity unknown in any other system (Rivers, Chapter 2).The lack of a visual dialogue between males and females necessitates a finely-tuned tracking and

interception of intermittent visual signals in three-dimensional space by females (Rivers, Chapter 4). The complexity and uniqueness of many aspects of the courtship behavior of *V. annecohenae*, coupled with our ability to observe it in controlled laboratory settings, has given us the opportunity to expand our understanding of the mating behavior in organisms that utilize luminescence for courtship and in crustacea in general.

REFERENCES

Andersson, M. (1994). *Sexual Selection*. Princeton, NJ: Princeton University Press.

Arak, A. (1983). Sexual selection by male-male competition in natterjack toad choruses. *Nature* **306**, 261-262.

Bailey, W.J., R.J. Cunningham, and Lebel, L. (1990). Song power, spectral distribution and female phonotaxis in the bushcricket, *Requena verticalis* (Tettigoniidae: Orthoptera): Active female choice or passive attraction? *Anim. Behav.* **40**, 33- 42.

Becker, P.H. (1982). The coding of species-specific characteristics in bird sounds. In *Acoustic Communication in Birds,* 1 (ed. D.E. Kroodsma and E.H. Miller), pp. 213-252. New York: Academic Press.

Beehler, B.M., and Foster, M.S. (1988). Hotshots, hotspots, and female preferences in the organization of lek mating systems. *Am. Nat., 131,* 203-219.

Bradbury, J.W., and Gibson, R.M. (1983). Leks and mate choice. In P. Bateson, ed., *Mate Choice,* pp. 109-138. Cambridge, UK.: Cambridge University Press.

Bradbury, J.W., Gibson, R.M., and Tsai, I.M. (1986). Hotspots and the dispersion of leks. *Anim. Behav.,* **34** 1694-1709.

Branham, M.A., and Greenfield, M.D. (1996). Flashing male wins mate success. *Nature*. **381**, 745-746.

Buck, J.B. (1988). Synchronous rhythmic flashing of fireflies II. *Q. Rev. Biol.* **63**, 265-289.

Cohen, A.C. (1983). Rearing and post-embryonic development of the myodocopid ostracode *Skogsbergia lerneri* from coral reefs of Belize and the Bahamas. *J. Crust. Biol.* **3(2),** 235-256.

Cohen, A.C. and Morin, J.G. (1986). Three new luminescent ostracodes of the genus *Vargula* (Myodocopida, Cypridinidae) from the San Blas region of Panama. *Contributions in Science, Natural History Museum of Los Angeles County* **373,** 1-23.

Cohen, A.C., and Morin, J.G. (1990). Patterns of reproduction in ostracods- a review. *J. Crust. Biol.* 10 (2): 184-211.

Cohen, A. C. and Morin, J.G. (2003). Sexual morphology, reproduction and bioluminescence in Ostracoda. In *Bridging the Gap: Trends in the Ostracode*

Biological and Geological Sciences. The Paleontological Society Papers (ed. L. E. Park and A.J. Smith), pp. 37-70, New Haven: Yale University Press.

Copeland, J., and Moiseff, A. (1997a). The effect of flash duration and flash shape on entrainment in *Pteroptyx malaccae*, a synchronic Southeast Asian firefly. *J. Insect Physiol.* **43 (10)**, 965-971.

Copeland, J., and Moiseff, A. (1997b). Intensity modulation and 'silent counting' in a Southeast Asian synchronously flashing firefly. *Biotropica* **29(1),** 126-128.

Cratsley C.K. and Lewis, S.M. (2003). Female preference for male courtship flashes in *Photinus ignitus* fireflies. *Behav. Ecol.* **14(1),** 135-140.

Darwin, C. (1871). *The Descent of Man, and Selection in Relation to Sex*. London: Murray.

Doherty, J. and Hoy, R. (1985). The Auditory Behavior of Crickets: Some Views of Genetic Coupling, Song Recognition, and Predator Detection. *Q. Rev. Biol.* **60 (4)**, 457-472.

Gerrish, G.A., and Morin, J.G. (2007). The life cycle of a bioluminescent marine ostracod, *Vargula annecohenae. J. Crust. Biol. Submitted.*

Gerrish G.A., Morin, J.G., Rivers, T.J., and Patrawala, Z. (2007). Darkness as an ecological resource: the role of light in partitioning the nocturnal niche *Oecologia in press.*

Hastings, W.J., and. Morin, J.G. (1991). Bioluminescence. Chapter 3 In *Neural and integrative animal physiology* (ed. C.L. Prosser), pp.131-170, New York: Wiley Interscience.

Herring, P.J. (2000). Species abundance, sexual encounter and bioluminescent signalling in the deep sea. *Philos. Trans. R. Soc. Lond, Ser. B.* **355 (1401),** 1273-1276.

Huvard, A.L. (1993). Ultrastructure of the light organ and immunocytochemical localization of luciferase in luminescent marine ostracods (Crustacea: Ostracoda: Cypridinidae). *J. Morph.* **218**,181-193.

Lewis, S.M., and Wang, O.T. (1991). Reproductive ecology of two species of *Photinus* fireflies. (Coleoptera: Lampyridae). *Psyche* **98**, 293-307.

Lloyd, J.E. (1966). Studies on the flash communication system in *Photinus* fireflies. *University of Michigan Museum of Zoology, Misc. Publications* **160,** 1-95.

Lloyd, J.E. (1975). Aggressive mimicry in *Photuris* fireflies: Signal repertoires in femmes fatales. *Science* **187,** 452-453.

Lloyd, J.E. (1980). Male *Photuris* fireflies mimic sexual signals of their female's prey. *Science* **210**, 669-671.

Lloyd, J.E., and Wing, S.R. (1983). Nocturnal aerial predation of fireflies by light-seeking fireflies. *Science* **222**, 634-635.

Markert, R.E., Markert, B.J., and Ventress, N.J. (1961). Lunar periodicity in spawning and luminescence in *Odontosyllis enopla. Ecology* **42(2),** 414-415.

Michaelidis, C.I., K.C.Demary, and Lewis, S.M. (2006). Male courtship signals and female signal assessment in *Photinus greeni* fireflies. *Behav. Ecol.* **17**, 329-335.

Michaud, T.C. (1962). Call discrimination by females of the chorus frogs, *Pseudacris clarki* and *Pseudacris nicrita. Copeia*. 1962, 213-215.

Moore, A.J. (1988). Female preferences, male social status, and sexual selection in *Nauphoeta cinerea. Anim. Behav.* **36(1),** 303-305.

Morin, J.G. (1986). 'Firefleas' of the sea- luminescent signaling in marine ostracode crustaceans. *Fla. Entom.* **69 (1)**, 105-121.

Morin, J. G. and Cohen, A. C. (1991). Bioluminescent displays, courtship, and reproduction in ostracodes. In *Crustacean Sexual Biology* (ed. R. Bauer and J. Martin), pp. 1-16. New York: Columbia University Press.

Morris, G.K., and Fullard, J.G. (1983). Random noise and congeneric discrimination in *Conocephalus* (Orthoptera: Tettigoniidae). In *Orthopteran Matin Systems: Sexual Competition in a Diverse Group of Insects* (ed. D.G. Gwinny and G.K. Morris), 73-96. Boulder, Colo: Westview Press.

Shuster, S.M. and Wade, M.J. (2003). *Mating Systems and Strategies*. Princeton, NJ: Princeton University Press.

Tsuji, F., and Hill, E. (1983). Repetitive Cycles of Bioluminescence and Spawning in the Polychaete, *Odontosyllis phosphorea*. *Biol. Bull.* **165 (2),** 444-449.

Vencl, F.V., and Carlson, A.D. (1998). Proximate mechanisms of sexual selection in the firefly *Photinus pyralis* (Coleoptera: Lampyridae). *J. Insect. Behav.* **11(2)**, 191-207.

Walker, T.J. (1983). Diel patterns of calling in nocturnal Orthoptera. In *Orthopteran Mating Systems: Sexual Competition in a Diverse Group of Insects* (ed. D.T. Gwynne and G.K. Morris) pp. 45-72. Boulder, Co: Westview Press.

CHAPTER 2

EXTREME PLASTICITY OF ALTERNATIVE MATING TACTICS AND CLASSIFICATION OF THE MATING SYSTEM OF A MARINE BIOLUMINESCENT OSTRACOD

Introduction

In mating systems where both female choice is possible and male-male competition is high, alternative mating behavioral tactics are common (Andersson 1994; Gross 1996; Shuster and Wade 2003). 'Tactics' here is defined as behaviors that can potentially be exhibited by all males in a population (genetic monomorphism), and is distinct from 'strategies,' which are considered behaviors restricted to that portion of the population that have the allele for that particular trait (see Gross 1996). The reversibility between alternative tactics is hypothesized to be highest when these tactics are under neuronal control, rather than hormonal or developmental control, as has been described in a variety of mating systems including scorpionflies, frogs, toads, and others (Thornhill 1981; Howard 1984; Sullivan 1983; see Shuster and Wade 2003). Alternative male mating tactics can include sneaking, satellites on territorial individuals, competing calls (such as synchronizing on an already displaying male), and female mimicry (Gross 1996; Shuster and Wade 2003). Rapid switching between tactics is expected when either the environment is highly variable within an individual organism's lifetime, thus favoring different behaviors in different situations (Levins 1968; Shuster and Wade 2003); it is also expected when the individual's condition or competitive situation varies on short time scales (Shuster and Wade 2003). The time scale of described systems where males "rapidly" switch between tactics is on the order of hours to days. Furthermore individual tactic changes often involve the dominant/calling male being removed from a courtship setting, opening up the

opportunity of a previous sneaker to start calling such as in green tree frogs (Perrill et al. 1978, 1982).

Vargula annecohenae (Crustacea: Ostracoda), a myodocopid ostracod in the family Cypridinidae, is found in great abundance in shallow seagrass beds of Belize. Males produce luminescent courtship displays in the water column above the seagrass beds in repeated, predictable, and complex patterns (Torres and Morin 2007 *in press*; Rivers Chapter 1). Starting during astronomical twilight, about 45 minutes after sunset or after moonset, whichever occurs last (Morin 1986; Morin and Cohen 1991; Gerrish et al., *in press*), males leave the benthos and enter the water column to secrete packets of luminescence, via nozzles in their upper lip, as a vertical array of light pulses. The displays consist of two phases: a bright, 'stationary' phase of 3-4 pulses, which acts to attract the attention of females and competing males, and a somewhat dimmer, predictable 'helical phase' of up to 16 pulses, which allows conspecifics to orient to, track and intercept the displaying male (Rivers Chapter 1) **(Figure 2.1)**. Both females and males are attracted to and approach these luminescent displays (Rivers Chapters 1, 3).

During the evening courtship period males either 1) initiate a display without input from other males [=displaying males] or they respond to the signals of other males 2) by tracking closely with the displaying male but without signaling [=sneaking] or 3) by producing parallel displays [=entraining]. The secondary entrainment responses are loosely synchronized with the other display. Morin (1986) has tentatively classified the mating systems displaying ostracods in the Caribbean as a temporal leks, also referred to as a spree (*sensu* Walker 1983).

Here we demonstrate that individual males exhibit any and all of these three highly plastic alternative mating tactics and switch among them over short time intervals of

seconds to minutes. We also indicate that the mating system of *V. annecohenae* is consistent with a lek mating system.

Methods

Field Experiments and Observations

Ostracods were collected by baited traps placed at night in seagrass beds or via sweep nets during the displays (as described by Rivers, Chapter 1).

Lab Experiments and Observations

Both individual behavior and luminescent signals were recorded during the displays produced by signaling males under dark conditions in the lab, but with infrared illumination in special laboratory tanks so that we could visualize individual male behaviors in the dark (see Rivers, Chapter 1 for details).

Analysis Protocol

Effect of male density on behavior: In the field, there are an average of about 2.5 males found in close proximity to a displaying male (Rivers Chapter 1). To determine the effect of density of males on the number of pulses per display, males were placed in a small, but tall (60cm [height] x 15cm [width] x 16cm [depth]) tank with the following male densities: 4, 5, 10, 20, and 30 males per trial. The numbers of pulses per display were then tallied from night vision display (NVD) recordings of 541 displays. To determine the effect of density on number of entraining males per display, males were placed in higher densities of 30, 50, 80, 110, 150, and 200 males per trial in the small tank, and the number of entrainments per display was determined from recordings of 335 displays.

Individual Male Behavior: First response and start tactic times and locations: Seven 30 minute trials were recorded between 22 April and 10 May 2003. The activity of five males, placed in the 60cm x 15cm x 16cm clear acrylic tank, was examined through frame-by-frame analysis of videos in Adobe Premiere Pro until we were no

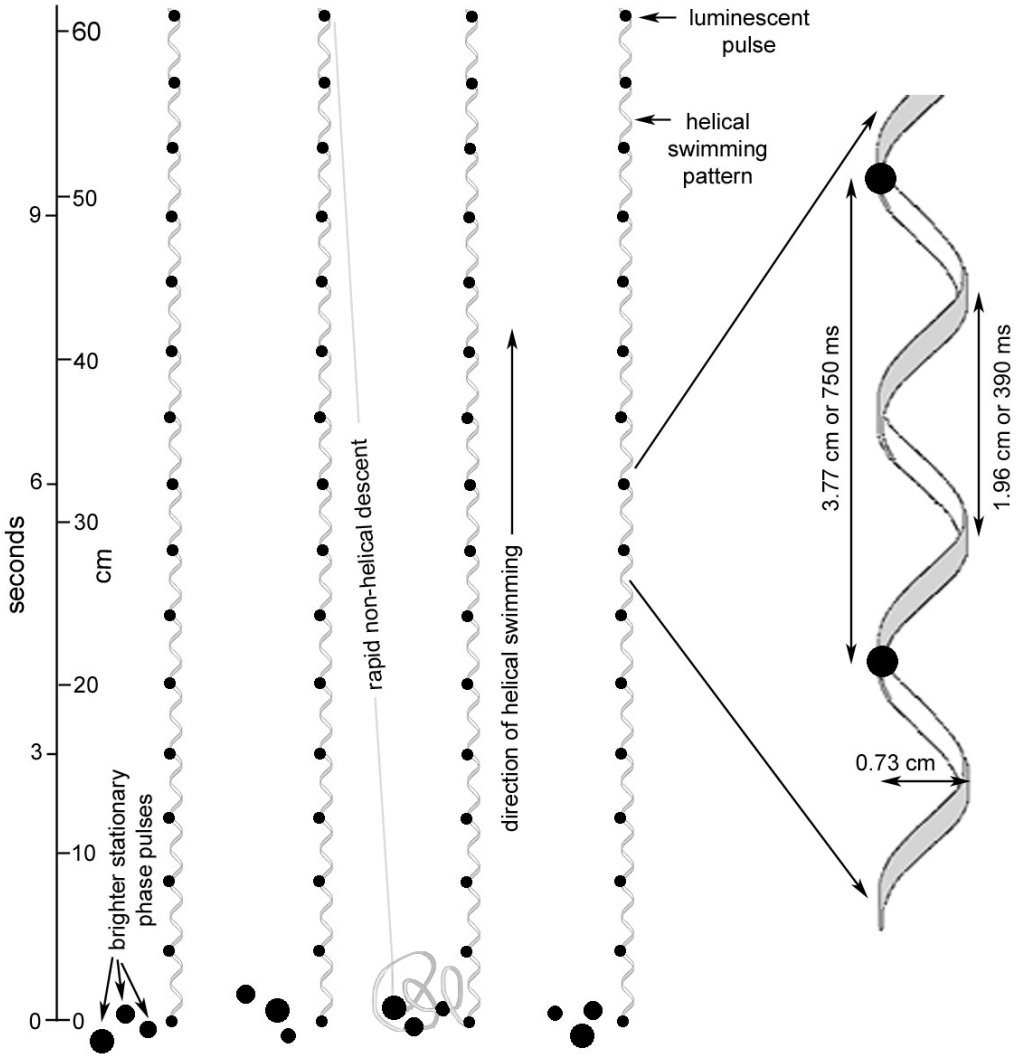


Figure 2.1. Model of 4 successive displays from a single male (From Rivers, Chapter 1)

longer able to differentiate individuals due to swarming or overlapping images of multiple males. Our first goal was to observe what mating tactics each of the five individuals employed, and in what frequency. If a male started a luminescent display on its own, we termed this tactic an 'initiating display.' If a male followed closely above the displaying male, but without luminescing on its own, we termed 'sneaking', and a male that synchronized his display with an initiating male was labeled as an 'entraining' male. We then calculated the percentages of time each male spent performing each tactic (initiating a display, entraining, and sneaking). We used a general linear model (SAS 9.1) to test whether there were any trends of entrainment percentages vs. sneaking percentages across trials, as well as whether the time a male had displayed during the trial had any effect on the alternative tactic chosen.

Male responses (N=187) to 85 displays were analyzed from 5 trials, each with 4 responding males, to determine what factors would predict which alternative tactic a male would employ to a given display. Frame grabs from digital video were taken for determining the time and location (distance and angle) of all males in the tank relative to a displaying male: 1) at the display start, 2) when the individual first responded to a display (with a distinct and discernable change in behavior, be it direction change, 'jerk,' or speed increase), 3) when the individual committed to starting a particular tactic and what tactic was chosen (there is a distinct change from a loose circling/approaching swimming style to a much more directed, focused swimming pattern), or 4) if the individual switched from entraining to sneaking. At each of these times, we also recorded the distance of the displaying male from the bottom, the pulse number within the display train, and whether the displaying male had started the helical portion of the display. A goodness-of-fit test was used on the numbers of tactics started before and after the helical portion of the displays. Distances and angles were calibrated and determined by using the Image-J analysis software. We also tested

whether the elapsed time of a start tactic, the elapsed time of 1st response, and the time after a pulse when a response or tactic occurred were predictive for the tactic chosen. In order to account for multiple measurements of individuals and to give more conservative error estimates than a simple logistic regression model, we used backward stepwise generalized estimating equations (GEE in SAS 9.1) to determine what aspects of distance or time could be used to predict the probability of entrainment.

Swimming speed analyses: To determine whether the speed of a male responding to a display differed from its 'cruising' speed, when no displays were present, we recorded the location of 8 males every 2 frames (1/15th of a second = 67ms) in a large but narrow 60cm (height) x 70cm (width) x 15cm (depth) clear acrylic aquarium. We defined a responding male as one that changed direction or apparent speed in order to approach a displaying male, but then examined its patterns before it started the alternative tactic. We used a paired-t test to determine whether differences were significant.

Seventy-nine clear displays and entraining displays from 5 of the 7 trials between 22 April to 10 May 2003 (in the small tank) were analyzed to ascertain the helical swimming speeds of males. We used two-dimensional video records in this analysis. We have previously confirmed that there is no significant difference between this method and actual 3-dimensional calculations (Rivers Chapter 1). We showed there that these swimming speeds accurately represent actual speeds. Male position (in X and Y coordinates) was recorded every 2 frames (1/15th of a second = 67ms), and point-to-point distances and speeds were calculated. Since the second part of the male display ("helical" or orienting phase) is a conserved helix, we calculated an actual helical swimming speed from the 2-dimensional data by determining the mean diameter of the helices, the apparent vertical speed, and the time it takes for a male to

complete each loop of the helix. To compare actual and vertical speeds between displaying and entraining males during a display and during the descent, we used a random effects mixed model (SAS 9.1) to account for multiple measurements from single individuals.

Results

Swimming Speeds: Non-displaying males sped up when responding to another male's signal. Males responding to a displaying male increased their swimming speed significantly from a cruising speed of 5.6 [+/- 0.38] cm/s to 8.6 [+/- 0.32] cm/s (N=8, t = -6.02179, p = 0.0002), an increase of 33.2% (**Figure 2.2)**.

Displaying males swam with a mean actual (as opposed to vertical) swimming speed of 9.2 [+/- 0.24] cm/s, while moving vertically with a mean rate of 5.7 [+/- 0.25] cm/s, and descended at a mean rate of 9.7 [+/- 0.25] cm/s [n=18]. Entraining males swam with a mean actual swimming speed of 8.7 [+/- 0.17] cm/s, while moving vertically with a mean rate of 4.8 [+/- 0.21] cm/s, and descended at a mean rate of 8.9 [+/- 0.28] cm/s [n = 12]. There was no significant difference in the interaction effects of tactic type and swimming type (f = 0.73, p =0.4879), most likely because the trends between entraining males and displaying males were quite similar (**Figure 2.3)**. Because we were interested more in the differences between tactics rather than the overall trends within the tactics, we hypothesized that there was biological importance in the variation between the initiator's and entrainer's tactics. Using a slice (SAS 9.1) to look only at the differences between descending speeds, actual display speeds, and vertical speeds between displaying and entraining males, we found that displayers swam at a significantly higher vertical rate than entrainers (f = 10.85, p = 0.0017), while there was no difference between their actual speeds (f =3.47,

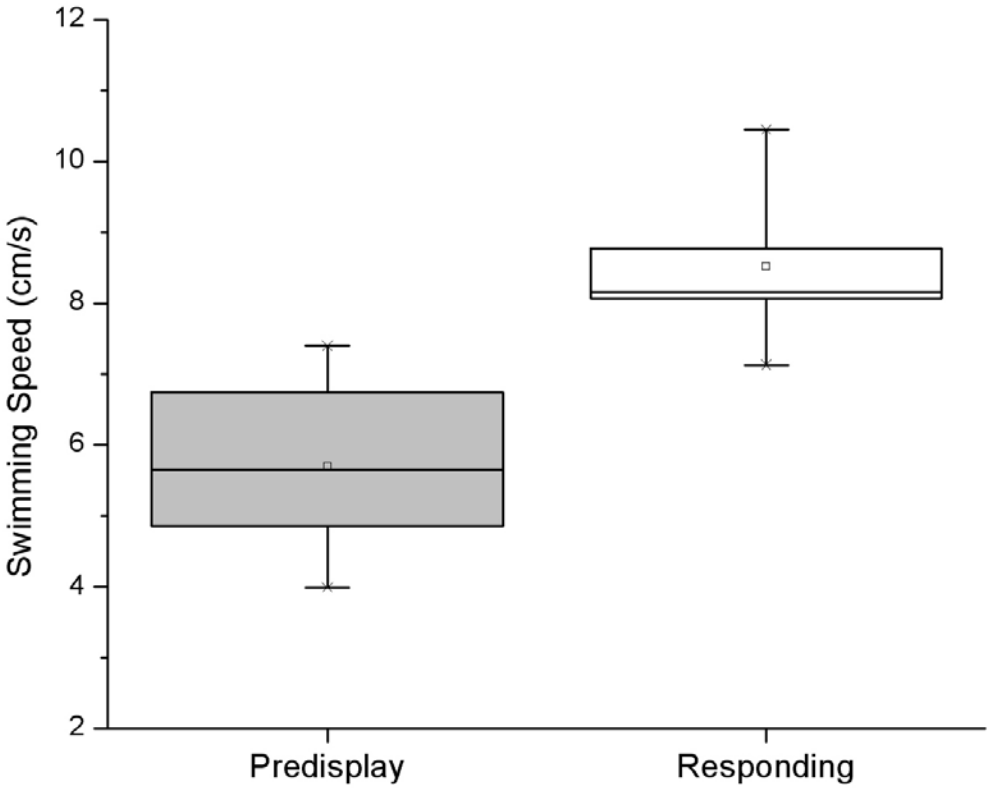


Figure 2.2. Change in swimming speed of responding males. Males increased their swimming speed by 33.2% when responding to another displaying male, but prior to starting an alternative tactic. The bars extend to the 95th percentile range, the box encompasses the 25th to 75th percentile range, the line shows the median, and the square in the center the mean.

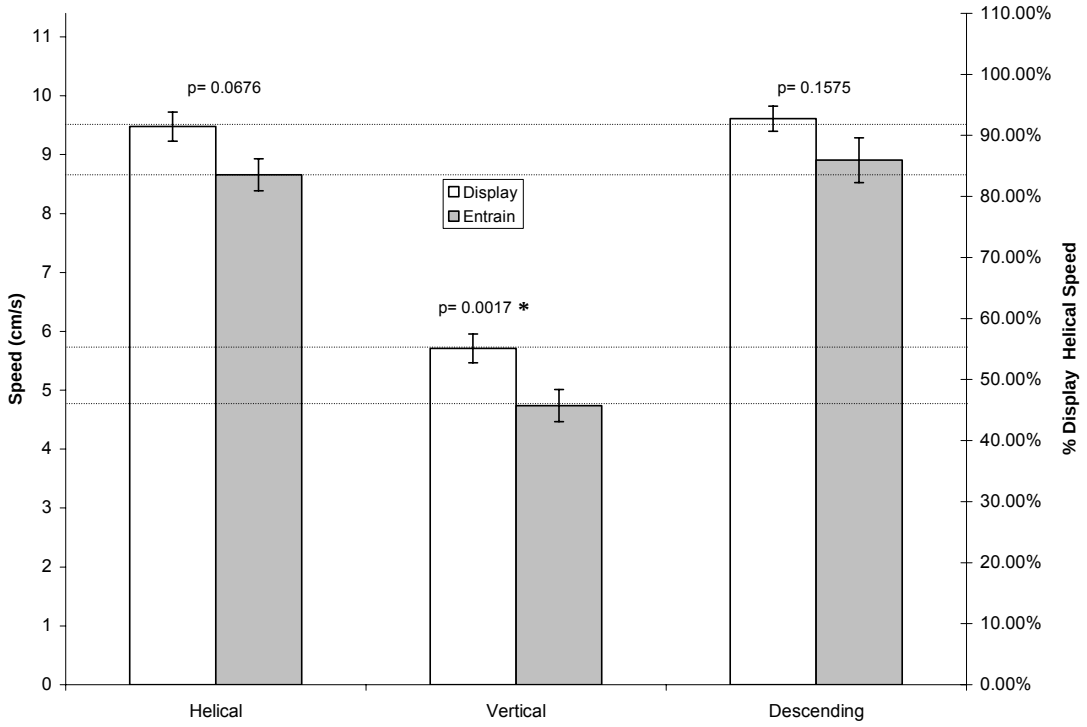


Fig 2.3. Comparisons of display and descending swimming speeds of displaying and entraining males. Helical swimming speed corresponds to actual swimming speed, while vertical corresponds to the vertical swimming speed component only. Males appear to slow their vertical speed more than their actual speed when entraining, indicating a change in pattern (wider spirals). The descending speed is the speed at which males descend toward the bottom of the tank after displaying. Bars are standard error.

p = 0.0676) or descent speeds (f =2.05, p =.1575)**(Figure 2.3)**. The data show that, while the actual (helical) swimming speed is not different from descent speed, the vertical speed, due to the helix, is only slightly more than half the descent speed.

Percent of activity spent during each tactic: In every trial, at least four out of the five males partook in courtship behavior. Of those participating, on average, individual males initiated displays 28 [+/-4.5] % of the time, entrained 30 [+/- 3.5] % of the time, and were sneakers 42 [+/- 3.6] % of the time. There were no significant differences between trials (F = 0.36, p= .8942), but there were significant differences among males within trials (F= 50.93, p <.0001). Certain males dominated displaying in each trial **(e.g., Figure 2.4).** When responding to another displaying male, individual males spent, on average 42 [+/- 3.8] % of their time entraining, and 58 [+/- 3.8] % of the time sneaking (N= 28), with significant differences between trials (f =8.74, p = 0.0003) and significant differences among males within a trial (F = 45.98, p < 0.0001). Males that spent more time displaying were more likely to sneak than entrain when performing an alternative mating tactic on another display (F = 9.84, p =0.007). For every increase in percentage of displaying, there was a 0.22% decrease in the probability of the alternative tactic being entrainment.

Effect of display phase on tactic start: The goodness-of-fit test, with a null hypothesis of equal probability of starting an alternative tactic during each phase, confirmed that the upward helical phase of a display was more important for predicting tactic start than the stationary phase, regardless of tactic type (χ^2 94.8122, p <0.0001). There were only 25 instances where an alternative tactic started during the stationary phase (but always after the pulse immediately preceding the helical phase), and 156 instances after the helical phase started. The proportion of alternative tactics chosen during the 25 instances of a tactic starting during the stationary phase was

Display	Male 1	Male 2	Male 3	Male 4	Male 5
1	♣	♠	♥		
2	♥	♠	♣	♠	♠
3	♣	♠	♥	♠	♠
4	♣	♦♥	♠	♥	♠
5	♥	♣	♥	■	♠
6	■	♦♥	♣	♥	♠
7	♥	♦♥	♣	♥	♠
8	♥	♣	♥	♠	♠
9	♦♥	♦	♣	♦♥	♠
10	♥	■	♣	♥	♠
11	♦	♣	♦♥	♦♥	♠
12	♣	■	■	■	♠
13	■	♥	♣	■	♠
14	♦♥	♥	■	♣	♠
15	♦♥	♣	♥	■	♠
16	■	♣	♦♥	♦♥	♠
17	♦	♣	♦♥	♦♥	♠
♣	4	6	6	1	0
♥	5	2	5	4	0
♦,♦♥	5	4	3	4	0
■	3	2	2	4	0
♠	0	3	1	2	16

♣= Displays
♥= Sneaks
♦= Entrains
♦♥= Entrains, then sneaks
■= Other- responds (change of movement) but then stops
♠= No reaction

Figure 2.4. One example [out of 7] of tactics exhibited by 4 active males and 1 inactive male during a 10-minute portion of a trial from 10 April, 2004. Columns correspond to individual male behavior per display. Boxes with two symbols show the male changed tactics from entraining to sneaking during that single display [Note: there were no instances of the reverse]. Male 5 showed no response during the course of the trial. The total number of times a male exhibited each tactic is summed at the bottom.

nearly equal (12 sneaking vs. 13 entraining), indicating no predictive value of display phase on tactic chosen.

Predictability of tactics: The times (elapsed time after the male first responds and elapsed time after each pulse) at which males responded to luminescent signals had no bearing on which alternative tactic was ultimately chosen. Neither the time at which males first respond to a display (χ^2 0.47, p =0.4924) nor tactic start times (χ^2 0.00, p =0.9721) had significant power to predict whether a male would sneak or entrain. Elapsed time after a pulse was also found to be insignificant and was taken out of the equation prior to the final model. However, the distance of a male exhibiting an alternative mating tactic from the displaying male has strong predictive power on the tactic chosen, with vertical distance being the strongest factor: the higher above a displaying male the initiator was, the more likely the responder would entrain rather than sneak **(Figure 2.5)**. When performing a backward-stepwise GEE, the responders' 1) location relative to the initiator when it first responded (X,Y, and interaction between them) **(Figure 2.6a)** and 2) whether the time a male spends displaying vs. sneaking or entraining, had no significant effect in predicting the probability of entrainment**,** so we also removed them from the final model. The final model then becomes:

Probability of Entrainment = -3.3463 + [0.3208x(Horizontal Distance, X)] + [0.4189x(Vertical Distance,Y)] - [0.273x(Interaction between X and Y)]

With an α level of 0.05, neither horizontal distance (χ^2 2.85, p = 0.0911) nor the interaction of horizontal and vertical distance (χ^2 3.5, p = 0.0614) was found to be significant in predicting whether an individual would entrain**.** Only vertical distance proved to be significant in predicting entrainment (χ^2 14.11, p =0.0115). However, all

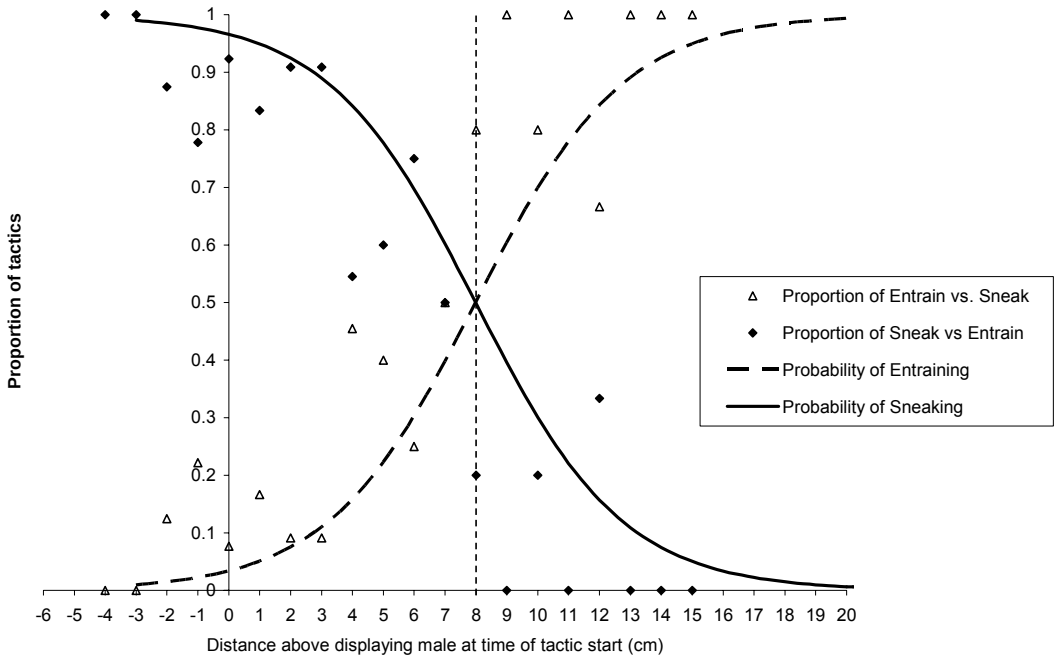


Figure 2.5: Proportion of males exhibiting either sneaking or entraining as a result of distance from a displaying male. Data points are the proportion of tactics at each distance from tracking individual males from 5 trials (N = 187), and lines are the predicted probability of each tactic as a result of our general estimating equation analysis. The point where there is equal likelihood of entraining or sneaking (vertical dashed line) is at 8 cm above the displaying male.

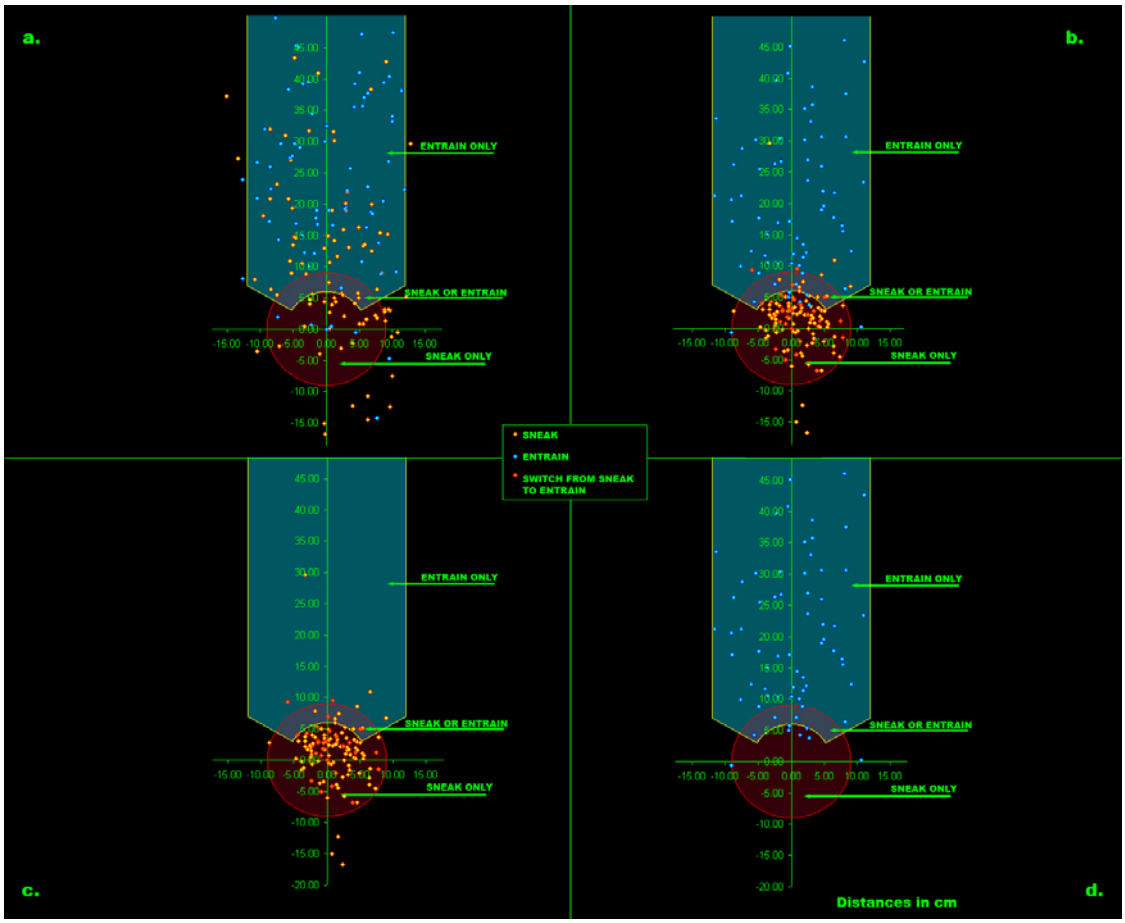


Figure 2.6. Location of males in relation to the displaying male. The color-coded areas correspond to the 95th percentile limits for 1) entraining males, 2) sneaking males, and 3) the overlap areas between the two. **a.) First response:** locations of males that entrain (blue) or sneak (yellow) when they first respond to a display. There was no significant predictive value as to which tactic an individual chooses based on 1st response location **b.)** locations of males when they start entraining, sneaking, or switch from entraining to sneaking (red) **c.)** locations of only males that initially sneak or switch from entraining to sneaking **d.)** locations of only males that entrain. The zero point on each graph is the location of the displaying male, not actual height in the water column.

distances were left in the final model, because their inclusion more closely matched the actual proportions of entrainers and sneakers from laboratory observations.

Density effects: The density of males had strong effects on both display length and number of entraining males per display. Increasing the density of males significantly decreased the number of pulses per display (N = 541, F 89.01, $p < 0.0001$). There was approximately one less pulse per display for every 5 males added, with an intercept of 12.15 pulses per display at zero. This yielded the following model:

Pulse number = 12.154 – [0.198x (number of males in the tank)]

Increasing the density of males also increased the number of males entraining per display (N = 335, F= 129.24, $p < 0.0001$), although the increase was not linear. For every male added, there was a square-root increase of 0.007 entrainments per display.

Discussion

Our observations of the mating behavior of male luminescent ostracods demonstrates that there is remarkable plasticity of mating behaviors within a single individual and indicates which factors contribute to the choice of employing a particular tactic. Each male *V. annecohenae* has the ability to perform every mating tactic. Any male participating in courtship behavior is capable of either initiating displays, entraining, or sneaking during the hour-long display period each night. Certain males displayed much more than others in laboratory trials, which could be due to the condition of the individual (such as differences in energy or luminescent chemical reserves (Rivers Chapter 3), as occurs in other animals exhibiting alternative mating tactics (Dawkins 1980; see Andersson 1995; Shuster and Wade 2003). However, there may also be intrinsic (genetic) differences among males, with some

more likely to display than entrain or sneak than others, even when all males are in similar condition. In assessing alternative mating tactics, one goal is to assign a fitness value for each of the tactics used. The extreme plasticity of this system makes it difficult to calculate a fitness payoff for each tactic, since one would have to know exactly which tactic a male was exhibiting just prior to successful copulation with a female. However, we have not yet observed successful copulation in the lab because the confines of the tanks and lack of refuges produce encounters with females even without displays. Until these issues are overcome and we can observe successful female choice and copulation under more natural conditions, we will be unable to assign fitness values for individual tactics.

Entrainment: Synchronous displays are widespread and common in many insects, anurans, and birds that use acoustic (see Greenfield 1994 a,b) or luminescent displays (Buck and Buck 1978; Case 1980; Carlson and Copeland 1985; Morin 1986; Buck 1988; see Greenfield 1994a, b). Synchrony can be either cooperative to increase mating success, or competitive, where males try to 'jam' the signals of competing males (Greenfield 1994b). In *V. annecohenae* where the luminescence and swimming patterns of entrainers are loosely synchronized with the initiator, the displays may be both cooperative and competitive. More luminescent displays in an area may draw in receptive females from greater distances, indicating cooperation, but upon arrival, different signals are likely competing for attention. A similar situation has been postulated for the spectacular group signaling firefly trees of Southeast Asia (see Buck 1988), and synchrony and competition may occur in North American fireflies as well (see Vencl and Carlson 1998). In *V. annecohenae,* males often change from sneaking on one luminescing individual, to another (one entrainer to another, an entrainer to a sneaker, or vice versa), thus indicating that males are able to discern between different displays. We hypothesize that the brighter displays may be more attractive to both

females and competitive males, and responding individuals will switch to following the brighter displayer.

Entrainment patterns are different from the patterns of initiator males in one important aspect: although entraining males are swimming about the same actual speed as displaying males, their vertical speed is significantly slower, which means they are swimming in a wider helical pattern than initiator males. This wider sweep may be an attempt to increase an entrainer's chances of being intercepted by or intercepting a female responding to an initiator and approaching from the side or below. If she was previously tracking toward a displaying male, it may benefit the entraining male to try to close the gap between him and the displayer, and thus be closer to the trajectory of an approaching female. The vertical speed of entraining males is close to that of the swimming speed of responding females, whereas displaying males are swimming vertically significantly faster than females (Rivers Chapter 4). An important observation is that a female appears to respond to a male only when the male signals below her position (Rivers Chapter 4). It is quite probable that because entraining males are even higher up in the water column when they start they may be level with or even above a responding female, and the only way to be intercepted by the female is by swimming vertically at a rate at which a female can overtake them.

Sneaking: We have observed multiple males switch tactics up to 3 times during a single, 15-second display. Both entraining and initiating males may switch to sneak on other displaying males. Sneaking males can switch from sneaking on an entrainer to sneaking on the original displaying male, or from one entrainer to another, or from an initial displayer to an entrainer. However, while sneaking males may change from one target to another, the converse does not happen: we have not seen a sneaker become an entrainer during a display. Males involved in switching tactics

were always approaching a luminescent signal, and since distance is a good predictor of what tactic is used **(Figures 2.5, 2.6)**, a male switching from sneaking to entraining would have to swim away from a display to widen the distance before commencing, thus potentially decreasing his probability of success. Sneakers must thus wait to be a signaler, either as an initiator or an entrainer, until the next display bout.

Time to decide: The behavior of males after they respond to a display, but before they commence distinct sneaking or entraining, may indicate a decision point. Such males increase their swimming speed when responding to a display (by contrast, females responding to a display do not change *their* swimming speed (Rivers Chapter 3)). These uncommitted males either approach the initiator directly or, if above him, swim in circles until the displayer approaches them before sneaking or entraining. This pattern, therefore, may be a 'fishing' tactic, where males may be sweeping for a nearby or approaching female with the intention of intercepting and copulating with her before the display gets close. Increasing his swimming speed may increase the male's chances of coming across a female or, potentially, her pheromone trail, if it exists. If so, we might consider this behavior as a tactic on its own, or the first phase of the alternative tactics of entraining and sneaking males.

The rapidity of tactic switching: Although alternative mating tactics are quite common in many mating systems (see Shuster and Wade 2003 for review), the rapidity of switching between tactics in *V. annecohenae* appears to be unusual. To our knowledge, these displaying luminescent male ostracods have the fastest alternative tactic switching behavior described to date. There are cases where males of other species switch from being silent satellites to displaying (calling) over the course of a night, or from sneaking to displaying over a longer course of time (Thornhill 1981; Arak 1983; see Shuster and Wade 2003); but to repeatedly change tactics within

seconds, and sometimes multiple times within a *singl*e display shows how plastic the male mating behavior is in these ostracods.

Since all males that switched from entraining to sneaking were within 10 cm of the displaying male, intensity, distance, and orientation of the neighboring pulses are likely the major cues that determine switching tactics in the presence of competing displays (**Figure 2.5b,c**). Because of the rapidity and type of tactic changes that occur, this situation suggests that all males, including the initiator males, are continuously measuring their surroundings (i.e. other displays), and will even change their behaviors mid-display to maximize their likelihood of intercepting a female depending on the circumstances. In addition to tactic switching, we have observed a few instances where individuals initiated a display and then either stopped displaying to sneak (if within15 cm), entrain (only if above), or subsequently ignore n a brighter-displaying male (if beyond 15cm). In these cases the initiator may be comparing itself to the entrainer and deciding that the entrainer, because of its better position, will be more likely to attract a female rather than the initiator itself. This failed initiator then will have to choose to either approach the entrainer to sneak, or wait in a position where it is more likely able to start displaying again before others do.

Mating System Plasticity: Other aspects of this system may provide additional clues for why there is such extreme plasticity in male mating behavior. First, there is a highly skewed operational sex ratio (>175 males per female) on the display grounds (water column), which makes ultimate female choice challenging, and, if present, highly specific (Rivers, Chapter 1). Second, males can be drawn to other displays from a distance of *at least* one meter, which was the maximum separation distance available in our aquaria. Third, responding males do not start an independent display close to an already displaying male (i.e. < 30 cm), and, if responding from below, the males always sneak, but if from above, they either entrain or sneak (Figure 3). These

rules put a limit on the amount of time an individual could display during a single night's courtship period (ca. 1 hour) as a consequence of other male behavior. If a male displays, then descends to display again but before he can start, another nearby male displays, then the 'interrupted' male could wait until the current male stops luminescing to start another display, but hopefully before another male starts. However, this waiting takes him out of the area where he would be most likely to encounter a receptive female for at least one display round. Alternatively, this male could entrain in an attempt to usurp a female's attention (if far enough above the displaying male), or sneak in an attempt to intercept a female before she encounters the displaying male. By participating in every possible display through the use of all three tactics that conditions allow, he is maximizing the likelihood of encountering a female. The long-accepted description of sneaker or satellite males in many systems is that they are 'making the best of a bad job,' and that they are at some fitness disadvantage to displaying or territorial males (Dawkins 1980). This dichotomy may result from age and size differences, which have been documented many different animals including anurans, insects, and elephant seals (Le Boeuf 1974; Borgia 1980, Arak 1983; see Andersson 1994). However, in the current case of *V. annecohenae,* which has determinate growth with little size variation between adult males (Gerrish and Morin, *in press*), such differences seem unlikely. For these ostracods, merely being in an area where another male has started, means, that to participate, the male must choose to sneak or entrain rather than display. In other words, each male is making the best of a range of opportunities in the face of stiff competition, rather than making 'the best of a bad job.'

How to choose a tactic: Of all the cues available to a male, the vertical distance from a displaying male at the time of tactic start **(Figure 2.5b)** seems to be the strongest predictive factor in tactic choice, while the distance from a displaying

male at the start of a display has no predictive value **(Figure 2.5a)**. At the time of the start of the tactic, horizontal distance and the interaction between vertical and horizontal distance were not significant at the $\alpha = 0.5$ level, but we suggest that they may still play a biologically significant role in tactic choice in males. By incorporating horizontal distance and the interaction of vertical and horizontal components in the final model, the predictive curve matches the observed data much closer than when the parameters were removed, thus indicating they play an important role in male tactic choice (**Figure 2.6)**. The model indicates that if a male is sneaking, one can reliably predict that it started that tactic within 10 cm of the displaying male, while if it is entraining it predicts that it started above the male and at least 10 cm away.

However, we do not yet know all of the factors contributing to *when* a male commits to starting a tactic, including displaying. For instance, a question remains whether males exhibit prior control over how far they are from another male before starting a tactic: is it random chance, or is there another unknown cue? Since there is strong correlation between the start of the helical phase of a display and the commencement of alternative mating tactics by other males, is this helical pattern the only real criterion for when to start a tactic? There may be other factors, including various conditions of the male, such as its display history and capacity to produce pulses, that are important, but are as yet unknown. For example, a male that has displayed heavily during previous display periods (nights), or a male that survived a predation attempt by releasing copious amounts of luminescence, may have diminished luminescent capacity in its secretory cells (see Huvard 1993; Rivers Chapter 4) and therefore will display less and sneak more. We found that there is a negative correlation between initiator display and entrainment (the more a male is found to initiate displays, the alternative tactic is more likely to be sneaking rather

than entrainment), which may lend support to this hypothesis. However, this relationship may also occur in males that had just previously displayed and wound up low in the water column due to their possible intent to display again. Before these individuals can initiate, though, they may be interrupted by another male's display, and if their location places them either within 10cm of the display or below the other male, thus leaving sneaking as the only alternative tactic.

Although we are able to predict the time at which the first displays will be observed in the field to nearly the minute based on time of sunset and cloud cover (Morin 1986; Morin and Cohen 1991; Gerrish et al., *in press*), we do not yet know what specific factors males cue on to initiate displays. The presence of conspecific males or females may be required, since we were unable to elicit displays with low (<4) densities of males in the lab. As in other systems (Plaistow and SivaJothy 1996; Shelley et al. 2002), energy reserves may also affect male courtship behavior. Underfed individuals may display or even respond to other displays less than satiated males. While any tactic carries an energetic cost, sneaking, which does not involve luminescent secretions, is likely to be the least costly, and if tactic choice is condition-dependent, then energetically depleted individuals should sneak more than display or entrain. External factors, such as intensity of the intitiating display and clarity of the water may influence the distance at which males respond and commit to a tactic. There is significant variation in display intensity (the brightest display in a trial was nearly 70% brighter than the dimmest), and we hypothesize that males will respond and react from farther distances if the display is brighter (Rivers Chapter 1).

Formation of display hotspots: The behavior of males responding to other displays in the lab may help explain the distribution of displays above the seagrass beds. In the field, low density display areas dominate over large areas, but ephemeral hotspots in apparently homogenous regions and predictable hotspots around coral

rubble also occur (Rivers, Chapter 1). Since males respond to a display from a distance of at least 1 meter in the lab, and based on the response speed of the males, we can calculate minimum response distances. A male swimming at the mean response speed of 8.25 cm/s (over 40 body lengths per second) could conceivably detect, respond to, and intercept a 15 second display from a starting distance of 1.3 meters, or over 650 body lengths away. Thus, ephemeral hotspots could form when multiple males, responding to an individual display from a considerable distance, converge, thus causing clustering. The higher density of males would then increase the number of entraining males, which then further attract more males to the area by increasing the amount of luminescence in an area. Why we still see areas of lower display densities, as well as hotspots, needs further investigation. A possible explanation is that males in the lower-density display areas are already responding to a display and therefore not approaching a hotspot, and that the hotspot luminescence is too dim due to distance to out-compete the near display.

The mating system: Multiple aspects of the interactions between males and females for reproduction can be used place the mating system of *V. annecohenae* within the framework of other mating systems (see Shuster and Wade 2003). There is no seasonal mating period in *V.annecohenae;* rather, courtship and mating is lunar-dependent and occurs throughout the year, with female receptivity being seemingly asynchronous (Gerrish and Morin, submitted; Cohen and Morin 1990). Male *V. annecohenae* displays are found in the water column *only* during display periods and for no other purposes (e.g., feeding) (Morin 1986; Morin and Cohen 1991; pers. obs). During about 95% of a diel cycle (about 23 out of 24 hours) these ostracods remain in or on the benthos. Unlike many displaying animals, who have strong territorial boundaries, male ostracods do not appear to aggressively defend identifiable territories. Rather, they respond to and participate in displays from large distances. In addition, a

single display may be displaced horizontally during a single display by about a half-meter by water currents, thus making display territory defense unlikely. We have evidence of both female tracking towards displays, as well as females resisting copulation attempts by males (Rivers Chapter 3), which suggests the presence of female choice. There is no evidence of post-copulatory resource provisioning or male parental care in these ostracods such as behavioral mate guarding or resource defense (Gerrish and Morin, in prep). Male *V. annecohenae do* leave a sperm plug covering a female's genitalia after copulation (Cohen and Morin 1990), but whether it contains nutrients that benefit the female is unknown. Unlike adult *Photinus* fireflies, which do not feed in the field as adults (Lloyd 1997; Williams 1917), *V. annecohenae* males and unmated females, but not brooding females, have been found to readily feed in laboratory settings. Consistent with these observations, we do not find brooding females in traps in the field (Gerrish et al*, in press*), suggesting that once mated, females, while brooding, reduce their feeding activity, if not abandoning feeding altogether. Therefore, it is possible that nutrients in a spermatophore may add significantly to the energy reserves of a female while brooding, such as occurs in some crickets and fireflies (Sakaluk 1986; see Hoglund and Alatalo 1995; Lewis et. al. 2004).

First described in birds, leks are found in a wide variety of other organisms (see Hoglund and Alatalo 1995; Shuter and Wade 2003). There are four conservative criteria that Bradbury (1981) puts forth for a mating system to be considered a 'classic' lek: 1) there is no male parental care, 2) the lek is in an area utilized for the sole purpose of courtship, 3) there is possible female choice, and 4) a female gains no significant resources from the male other than gametes. Although quite common in insects, the closest account of classical lekking behavior in non-insect arthropods is in the fiddler crab *Uca sp.* (Croll and McCLintock 2000). However, in this case, males

violate the assumption of resource independence by guarding the burrows while females incubate for a few days and thus does not appear to be a true lek. *V. annecohenae* also resembles a classic lek, although we would not be able to definitively classify it as such without further research and determining the functions of the spermatophore.

Conclusion: Our observations of the male behavior of luminescent ostracod *V. annecohenae* have expanded significantly our understanding of the plasticity of mating tactics and indicate that individuals can change tactics rapidly to a level not previously known. Although extremely plastic, there appear to be fairly simple rules that, when followed, allow us to predict with high accuracy which alternative mating tactic will be chosen under particular circumstances. The small size of ostracods, coupled with our ability to observe and record their behaviors in the laboratory, allows us to manipulate external and internal conditions of individuals so that we can further dissect the nuances of the male mating behavior. Their male mating behaviors, as well as previous morphological and life-history studies (Morin and Cohen 1990; Morin and Gerrish in prep) and recent discoveries of female responses to luminescent displays (Rivers Chapter 4), lead us to conclude that the mating system of *Vargula annecohenae* is closest to a lek mating system.

REFERENCES

Anderson, M. 1994. *Sexual Selection.* Princeton, N.J.: Princeton University Press,

Arak, A.1983. Sexual selection by male-male competition in natterjack toad choruses. *Nature* 306: 261-262.

Borgia, G. 1980. Sexual competition in *Scatophaga stercoraria*: Size- and density-related changes in male ability to capture females. *Behaviour*. 75:185-205.

Bradbury, J.W. 1981. The evolution of leks. In: *Natural Selection and Social Behavior* (Ed. by R.D. Alexander and D.W.Tinkle), pp. 138-169. New York: Chiron Press.

Buck, J. 1988. Synchronous rhythmic flashing of fireflies II. *Q. Rev. Biol.* 63: 265-289.

Buck, J., and Buck, E.1978. Towards a functional interpretation of the synchronous flashing by fireflies. *Am. Nat.* 112: 471-492.

Carlson, A. D. and Copeland, J. 1985. Flash communication in fireflies. *Q. Rev. Biol.* 60:415-436.

Case, J. 1980. Courtship behavior in a synchronously flashing, aggregating firefly, *Pteroptyx tenor. Biol. Bull.* 110: 613-625.

Cohen, A.C., and Morin, J.G. 1990. Patterns of reproduction in ostracods- a review. *J. Crust. Biol.* 10 (2): 184-211

Croll, G.A. and McClintock, J.B. 2000. An evaluation of lekking behavior in the fiddler crab *Uca* spp. *J. Exp. Mar. Biol.* 254(1): 109-121.

Dawkins, R. 1980. Good strategy or evolutionary stable strategy? In G.W. Barlow and J. Silverberg (eds.). *Sociobiology: Beyond Nature/Nurture?* pp. 331-367. Boulder, CO: Westview Press.

Gerrish G.A., Morin, J.G., Rivers, T.J., and Patrawala, Z. *in press.*

Greenfield, M.D. 1994a. Cooperation and conflict in the evolution of signal interactions. *Annu. Rev. Ecol. Syst*. 25: 97-126.

Greenfield, M.D. 1994b. Synchronous and alternating choruses in insects and anurans: common mechanisms and diverse functions. *Am. Zool.* 34: 605-615.

Gross, M.R. 1996. Alternative reproductive strategies and tactics: Diversity within

sexes.*Trends Ecol.Evol.* 11:92-97.

Hoglund, J., and Alatalo, R.V. 1995. *Leks.* Princeton University Press, Princeton, NJ

Howard, R.D. 1984. Alternative mating behaviors of young male bullfrogs. *Am. Zool.* 24: 397-406.

Huvard, A.L. 1993. Ultrastructure of the light organ and immunocytochemical localization of luciferase in luminescent marine ostracods (Crustacea, Ostracoda, Cypridinidae). *J. Morph.* 218 (2): 181.

Levins, R. 1968. *Evolution in Changing Environment.* Princeton, NJ: Princeton University Press.

Le Beouf, B.J. 1974. Male-male competition and reproductive success in elephant seals. *Am. Zool.* 14:163-176.

Lewis, S.M., Cratsley C.K., and Rooney, J.A. 2004. Nuptial gifts and sexual selection in *Photinus* fireflies. *Int. Comp. Bio.* 44 (3): 234-237.

Lloyd, J.E. 1997. Firefly mating ecology, selection and evolution. In: J.C. Choe and BJ Crespi, eds., *The evolution of mating systems in insects and arachnids*. pp. 184-192. Cambridge: Cambridge University Press.

Morin, J.G. 1986. 'Firefleas' of the sea- luminescent signaling in marine ostracode crustaceans. *Fla. Entom.* 69 (1): 105-121.

Morin, J. G. and Cohen, A. C. 1991. Bioluminescent displays, courtship, and reproduction in ostracodes. In: R. Bauer and J. Martin, eds., *Crustacean Sexual Biology*, pp. 1-16. New York: Columbia University Press.

Perril, S.A., H.C. Gerhardt, and Daniel, R. 1978. Sexual parasitism in the green treefrog (*Hyla cinerea). Science* 200: 1179-1180.

Perril, S.A., H.C. Gerhardt, and Daniel, R. 1982. Mating strategy shifts in male green treefrogs (*Hyla cinerea)*: an experimental study. *Anim. Behav.* 30: 43-48.

Plaistow, S. and SivaJothy, M.T. 1996. Energetic constraints and male mate-securing tactics in the damselfly *Calopteryx splendens xanthostoma* (Charpentier) *Proc. R. Soc. Lond., B.* 263 (1374): 1233-1239.

Sakaluk, S.K. 1986. Sperm competition and the evolution of nuptial feeding behavior in the cricket *Gryllodes supplicans* (Walker). *Evolution*. 40: 584-593.

Shelly, T.E., Kennelly S.S., and McInnis, D.O. 2002. Effect of adult diet on signaling activity,mate attraction, and mating success in male Mediterranean f ruit flies (Diptera : Tephritidae) *Fla. Entom.* 85 (1):150-155.

Shuster, S.M. and Wade, M.J. 2003. *Mating Systems and Strategies*. Princeton, NJ: Princeton University Press.

Sullivan, B.K. 1983. Sexual selection in the great plains toad (*Bufo cognatus*). *Behaviour* 84: 258-264.

Thornhill, R. 1981. *Panorpa* (Mecoptera: Panorpidae) scorpionflies: Systems for understanding resource-defense polygyny and alternative male reproductive efforts. *Annu. Rev. Ecol. Syst.* 12: 355-386.

Torres, E.A. and Morin, J.G. *in press.*

Vencl, F.V. and Carlson, A.D. 1998. Proximate mechanisms of sexual selection in the firefly *Photinus pyralis* (Colepotera: Lampyridae). *J. Insect Behav.* 11(2): 191-207.

Walker, T.J. 1983. Diel patterns of calling in nocturnal Orthoptera. In D.T. Gwynne and G.K. Morris, eds., *Orthopteran Mating Systems: Sexual Competition in a Diverse Group of Insects*, pp. 45-72. Boulder, Co: Westview Press.

Williams, F.X. 1917. Notes on the life-history of some North American Lampyridae. *J. NY Entomol. Soc.* 25: 11-33.

CHAPTER 3

FINDING MR. RIGHTS: FEMALE TRACKING OF COMPLEX INTERMITTENT LUMINESCENCT MALE MATING DISPLAYS IN A TINY MARINE OSTRACOD CRUSTACEAN

In mating systems where males utilize species-specific luminescent courtship displays to attract females, describing female response behavior has been restricted to only a few firefly species. Although luminescence is more prevalent in marine than terrestrial environments (Hastings and Morin 1991), behavioral descriptions of marine bioluminescence, especially in courtship, are rare. Male cypridinid ostracods (ca. 2mm) utilize luminescence in the most unique and complex displays known in marine environments (Morin 1986; Herring 2000). Females are hypothesized to be attracted to, and exhibit choice between, these signals (Morin 1986; Morin and Cohen 1991). However, their small size and night-time mating activity necessitates using laboratory experiments to observe individual behavior. Previous attempts to observe female behavior were confounded because males in a restricted tank intercepted the females before they could exhibit choice. Males, without displaying, would attempt, albeit unsuccessfully, to copulate with the female. Using a blue light-emitting diode (LED) array to mimic a male display in conjunction with infra-red (IR) cameras to record behavior, we provide evidence that females are responding to the pulsed light and approach the intermittent signal in such a way as to intercept an upwardly-signaling male from above. In order to maximize her proximity to the next pulse in the display, responding females that are above the males swim more directly, swim at more directed angles, and compensate their vector more to intercept the male above each preceding pulse than control females. These data indicate that luminescence is the primary signal to attract receptive females.

The majority of studies on how organisms intercept moving visual targets have been performed on mammals (with the vast majority on humans) (Lenoir et al. 1999; Port et al. 1997), and insects, especially in courtship in flies (Boedekker and Egelhoff 2003; Collett and Land 1978) and in prey capture (Olberg et al. 2000; Gilbert 1997). In all of these studies the target signal is continuous: although the signal may be observed intermittently by the tracking individual (for example, by a loss of contrast between the target signal and background), the actual signal itself does not disappear. The only study on truly intermittent visual signals utilized for tracking and interception has been done on *Photinus* fireflies that use luminescence for aerial guidance to intercept and consume extraspecific firefly males (Lloyd and Wing 1983).

Ostracods (Crustacea, Myodocopida, Cypridinidae) in the Caribbean exhibit spectacular, species-specific luminescent courtship displays at dusk (Morin1986; Cohen and Morin 1990; Morin and Cohen 1991). One abundant species, *Vargula annecohenae* displays above shallow (1-10m depth) seagrass beds off Southwater Caye, Belize (16.801^{o}N Latitude, 88.083^{o} W Longitude)(Torres and Morin, *in press*). The mating system of these ostracods is complex (Morin 1986; Cohen and Morin 1990; Herring 2000). Commencing about 45 minutes after sunset and for the succeeding hour, males exhibit vertical displays (up to 60 cm above the top of seagrass) that consist of up to 19 blue pulses of light secreted out of nozzles in the males' upper lip. The display has two phases. The first phase consists of 3-4 short (400 ms), bright pulses with some interpulse interval variation (0.9-1.3s), but with little upward movement (< 2 cm total) at or just above the top of the grass. The second phase then immediately follows when the male swims upward with as many as 16 shorter (200 ms), dimmer pulses with more consistent intensity and interpulse distances of about 4 cm and interpulse intervals of about 700 ms (Rivers Chapter 1). Each pulse is left behind the rapidly-swimming, spiraling male as a discrete packet of light in the water

column. Each pulse, by itself, gives little information on the directionality and speed of a displaying male, but taken together, the pulses yield information on the distinct, intermittent trajectory.

Other competitor males exhibit two alternative behaviors with respect to the displaying male: they may entrain by producing their own competing, similar luminescent pattern nearby and in loose synchrony with the initiator, or they may sneak by swimming above and close to the displaying male but without luminescing (Morin 1986; Rivers Chapter 2). A single male is capable of initiating, entraining, and sneaking, and they switch rapidly between tactics during the hour-long display period (Rivers Chapter 2).

Unlike fireflies, where there is a luminescent call-and-response 'dialogue' between males and females (Lloyd 1966), in *V. annecohenae,* females do not engage in any kind of luminescent duetting (Morin 1986, Morin and Cohen 1991). This lack of luminescence by females is more like the auditory mating systems, such as crickets and frogs (Wagner and Reiser 2000; Greenfield 1994) than other systems utilizing light as a signal. We hypothesize that by remaining dark females can exhibit more choice in the courtship arena (water column) where the operational sex ratio is highly-skewed toward males (>175:1)(Rivers Chapter 1) so that they do not attract overwhelming male attention. The lack of dialogue suggests that the female, of necessity, has a highly-developed and species-specific swimming behavior in order to be able to intercept and copulate with her target male, without having to rely on any initial behavioral change in a displaying male.

There are a number of aspects of this system that make it tractable for analyzing mating behavior: 1) *Vargula annecohenae* are sexually dimorphic with the larger females brooding and producing crawl-away juveniles (there is no planktonic

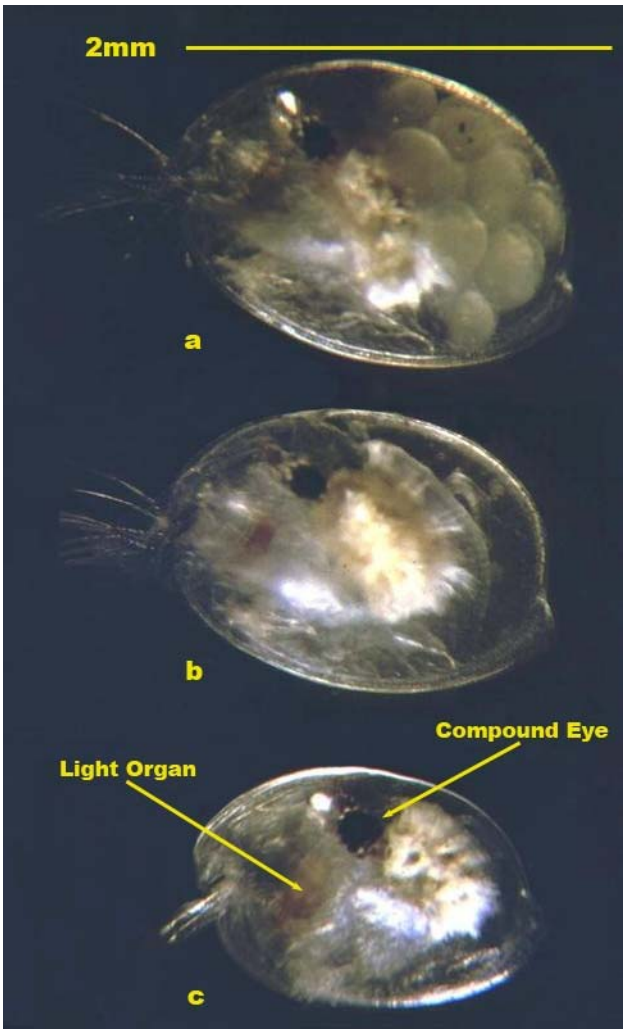


Figure 3.1. a. brooding female with embryos. b. adult female. c. adult male. *Vargula annecohenae* is a sexually dimorphic mydocopid ostracod found in great abundance in shallow marine grassbeds off Southwater Caye, Belize. Females brood young to juvenile (1st instar) stage; there is no planktonic larva. We are able to differentiate sexes in the 5th instar (A-1) stage, which allows us to isolate females and rear them as virgins for our experiments. All life stages are able to luminesce (for antipredation purposes), but only the males display for courtship purposes.

larval stage) **(Figure 3.1)**(Torres and Morin, *in press*); 2) it is possible to collect thousands of individuals in a single night in baited traps; and 3) males exhibit apparently normal luminescent courtship behavior in aquarium tanks in the lab. However, until now their small (ca 2mm) size and nocturnal activity have been prohibitive for answering more detailed questions about their behavior. Although infrared (IR) light attenuates rapidly in seawater, in laboratory tanks it is sufficiently reflected off the carapace of ostracods so that we can observe and record individual behavior, while at the same time not interfering with the male's ability to display. However, when we combined males and females in a tank, the confined spaces and absence of refuges for females allowed males to find them and attempt to force copulations prior to any female choice or tracking behavior. Since no broods resulted from these attempts we conclude that they were unsuccessful. Attempts to follow males and females placed in separate tanks were also unsuccessful, for the IR light levels necessary for tracking females throughout a large (70cm [height]x 60cm [width] x 15cm [depth]) laboratory tank overpowered the display of luminescence. This arrangement meant that only the first few pulses of a male display were visible on video, and we were unable to determine whether or not a female was reacting to luminescence. Therefore, in order to allow unhindered observation of female behavior to light signals that we are able to detect with our cameras, we designed a blue light-emitting diode (LED) array that mimicked male courtship luminescence patterns in space, time and pulse characteristics. The mean average pulse durations, interpulse intervals, and interpulse distances of ten natural male displays in the lab were used to construct the LED array "code."

We analyzed the swimming patterns of eleven virgin females that indicated distinct behavioral responses to the LED display by showing a change in swimming direction, angle, or speed. The virgin females were allowed to swim freely in a 70cm

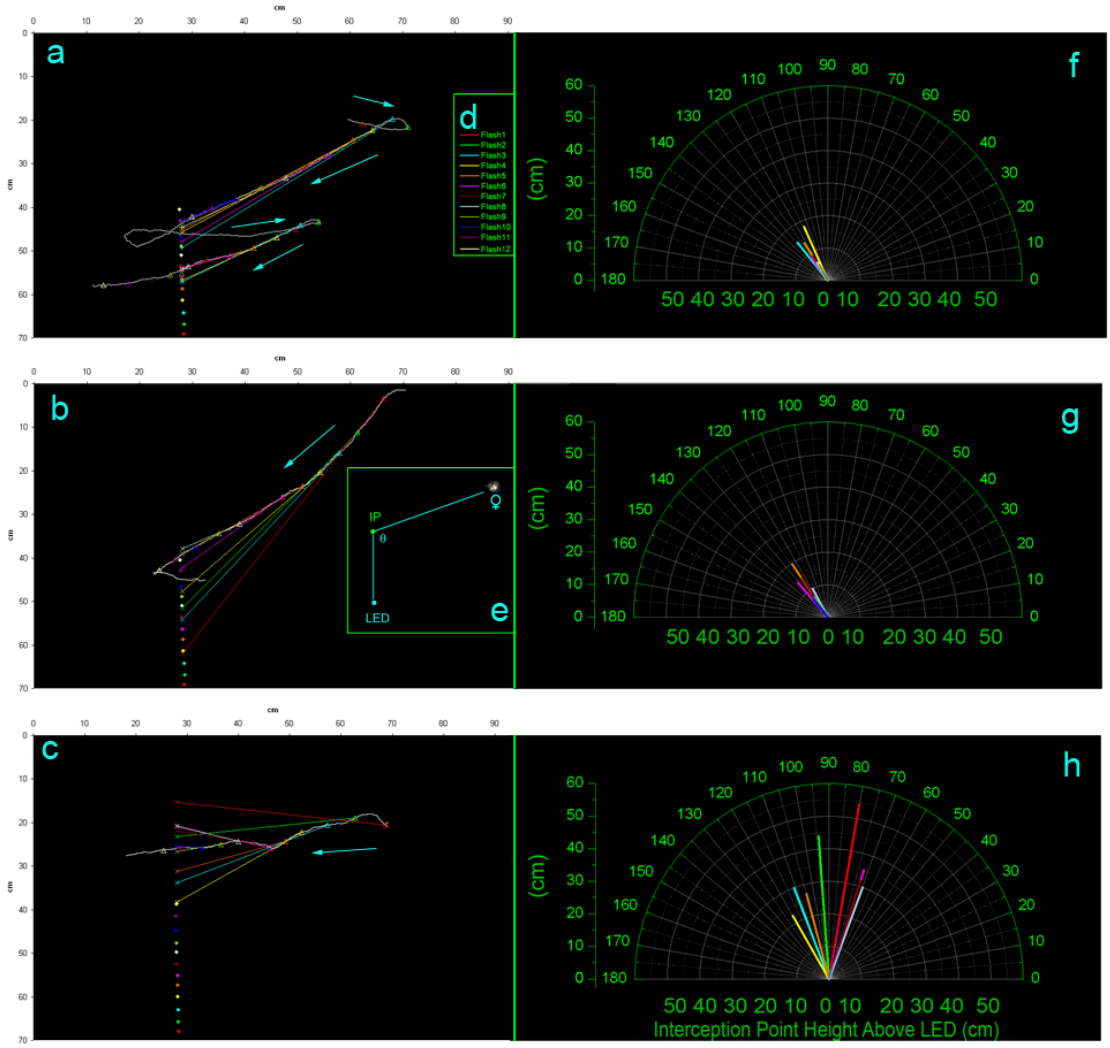
(height) x 60cm (width) x 15cm (depth) clear acrylic tank with the LED array adjacent to the left back wall. As a control, we analyzed 14 randomly-chosen swimming patterns of females when no LED displays were being emitted. In order to maximize observable behaviors free of wall-interference prior to a female intercepting the LED display, we restricted our analyses to only those females (both experimental and control) that were swimming from right to left toward the displays. In a typical 15-minute display period, 27 potential responses to luminescence were observed in females moving in this pattern. In contrast, in 15 minutes of dark, non-display periods, only 3 swimming patterns that at least superficially resembled responses to luminescence were observed.

When an LED display commenced, females that were initially swimming away turned to track the light signal after the 2nd or 3rd pulse (**Figure 3.2a**). A single pulse of light was not sufficient to trigger a female response. Subsequent pulses produced further changes in direction as females sought to orient toward the display **(Figure 3.2)**. Following the reorientation toward the display, responding females consistently oriented and moved toward the display (in both angle and linearity) compared to control females **(Table 3.1a, b)**. Between pulses, females responding to a display always swam so that they shortened the distance to the display, while control females swam variably, often increasing the distance from the display (**Figure 3.2**). Note that in order to provide the most conservative indication of behavioral changes, we chose only those (control and responding) females whose orientations and swimming directions were approaching the display, and because the displays are produced vertically upward, it follows that both experimental and control females showed a decrease in the interception point height above the previous LED (**Figure 3.3, Table 3.1c**.). However, the data clearly show that the rate of decrease of interception point distance is lower in responding females compared to control females **(Figure 3.3)**.

This distinct difference indicates that responding females are continually compensating their swimming vector to intercept the train above the next pulse, thereby ensuring that when she finally does intercept the display, she will be within a 2-3 centimeters of the next pulse (**Table 3.1c, Figure 3.2a,b**). The difference between the response trajectory of responding and control females is demonstrated by the difference in the angle of the female to the LED, with the interception point as the node **(Figure 3.2f,g,h).** The mean angle of control females was 90.1 (+/- 1.52, N = 129) degrees, which corresponds to a nearly flat swimming trajectory (slope of 0 degrees), while the mean angle of responding females was 110.7 (+/- 1.63, N = 60) degrees, showing responding females swim at nearly-constant 20 degree (to the LED) downward slope during the entire time she approaches. This constant correction to a consistent angle is reminiscent of how moving humans approach and intercept moving targets in 2-dimensional space (Lenoir et al. 1999). Because an individual luminescent pulse from a male *V. annecohenae* display demonstrates no directionality or speed of the displaying male, either 1) the female is predisposed to aim her trajectory above any single luminescent pulse at a particular angle, or 2) the female calculates her trajectory from the information given from multiple pulses. Since females do not significantly change their orientation until after the 2nd or 3rd pulse, multiple pulses are at least necessary. However, the question remains whether, once attracted, a female integrates information from multiple pulses to calculate interception, or she responds only to one pulse at a time.

Four criteria must be met, in proper sequence, to make the most conservative determination of female response to luminescent signals: 1) the female must ultimately be swimming in the direction of the luminescence display, either by already being correctly orientated, or by changing direction after the train commences; 2) the calculated interception points must be at or above the previous pulse in the train; 3) the

Figure 3.2. Examples of swimming patterns and trajectory angles of 2 responding females (top 2) and 1 control female (bottom). a: a female responds to 2 different displays, turning around after pulse 2 (green) both times. **b:** another female responds to a display. **c.** A typical swim path of a non-responding female. Color-coded triangles indicate the position of the female when the corresponding blue light pulse occurs (i.e. a red triangle corresponds to the red diamond, pulse 1). Colored lines in **a-c** mark the vector (allowing for time for swimming correction) of a female following each pulse if the female is swimming in the direction of the LED. Where the vector crosses the vertical display line is the interception point; the distance between this point and the preceding pulse is the interception point distance. See **Table 1c** for results and statistics. **d)** Colors correspond to the female response to a pulse number in **f-h. e)** The angles shown in **f-h** correspond to the angle between the female and LED, with the interception point (IP) as the node. A 90 degree trajectory indicates that the female is swimming horizontally; for anything below 90 degrees, she is swimming away from the LED, and for anything above 90 degrees, she is swimming toward the LED. The lengths of the colored lines indicate the distance between the IP and the LED. **f-h)** correspond to the angles of the female in figures **a-c** respectively. The trajectory of a responding female **(f,g)** is much closer to the LED pulse and with much less angle variation than in control females (**h**).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50 40 30 20 10 0 10 20 30 40 50
Interception Point Height Above LED (cm)

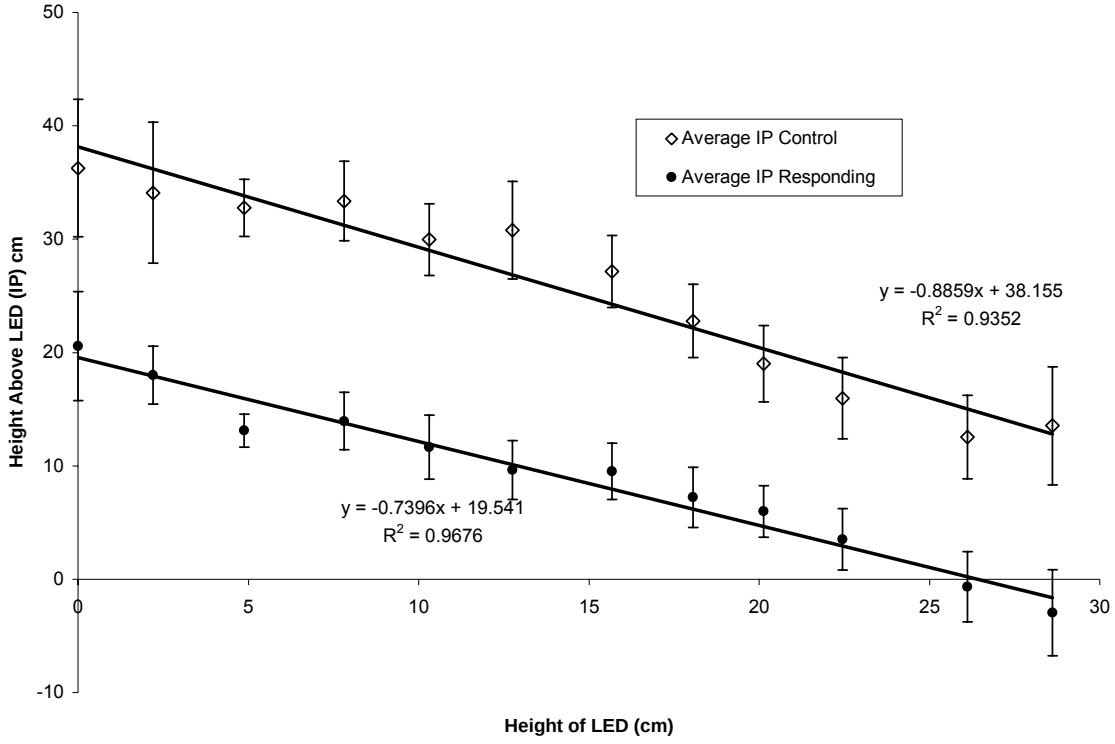

Figure 3.3. Height of mean interception point above the LED pulse by responding (solid) and control (open) females. Bars are standard error. The LED mimic display starts near the bottom of the tank and terminates almost 30 cm above the bottom. Both control and responding females show a decrease in interception point height as the display progresses, because each pulse of the mimic display is higher in the water column. The slope of responding females (-0.74) is significantly lower than the slope of control females (-0.89), indicating responding females are compensating their trajectory after each pulse.

Table 3.1. Swimming analyses of *Vargula annecohenae* females. Statistical results of 5 analyses, separated by horizontal lines, show the means, if applicable, the degrees of freedom (DF), t-value (t), p-value, sample size (n), and the model used for the analysis.

		DF	t	p-value	n	Model
a	**Total Swimming Slope**					
	Responding females swim at a significantly steeper slope (mean slope -0.48) than control females (mean slope -0.032)	24	4	**0.0006**	25	General Linear Model (GLM)
b	**Total Swimming Linearity (r-square)**					
	Females responding to LEDs swim significantly more linearly (mean r2 =0.957) during the time period of a display than control females (mean r2 = 0.48)	24	-4.28	**0.0004**	25	2sampleT
c	**Interception Point Height(Distance)**					
	Effect of pulse height	18.6	3.25	**0.003**		
	Interception point distances decrease significantly as pulse height increases				25	
	Effect of horizontal distance (X) of female to the LED	97.1	1.17	0.2453	(234 Obs)	
	X has no effect on interception point distance					
	Effect of vertical distance (Y) of female to LED	83.3	-0.42	0.6787		
	Y has no effect on the interception point distance					Random Coefficient
	Effect of interaction between pulse height and horizontal distance (X)	37.7	-2.12	**0.0408**		Mixed
	As X and pulse height both increase, there is a significant decrease in interception point distance					
	Effect of interaction between pulse height and vertical distance (Y)	21.9	7.1	**<.0001**		
	As both Y and pulse height increase, there is a significant increase in interception point distance					
	Effect of Treatment					
	The interception point distances are significantly closer to the pulses in responding females	23.9	3.95	**0.0006**		
	Effect of the interaction between pulse height and treatment					
	The rate at which interception point distances are reducing per pulse is slower in repsonding females (i.e. females are correcting their swimming vectors)	18.1	-2.78	**0.0123**		
d	**Linearity of Swimming between pulses (R-Square)**					
	Effect of pulse Height	248	-2.19	**0.0295**	25	Random Effects Mixed
	Swimming linearity (r-square) decreases 0.01 for every cm increase in pulse height				(264 obs)	
	Effect of Treatment	25	-3.36	**0.0025**		
	Responding females are swimming significantly more linearly between pulses					
e	**Swimming Speeds**	20	-0.167	0.869	25	2SampleT
	The mean swimming speed of responding females (4.53 cm/s) is not significantly different than the mean swimming speed of control females (4.58 cm/s)					

female must show compensation by changing her trajectory toward the display to close the distance between the female and the successive pulses; and 4) the female responding to a display should follow a nearly linear, non-erratic trajectory between each pulse (after allowing for the course correction). Deviation from one or more of these criteria would indicate that the female swimming pattern may not be a response to the luminescence. Using these strict criteria we were able to confirm more than 50 responses of females to luminescence across multiple trials. We were also able to reject the 3 swimming patterns of the control females that superficially resembled female responses, because that they violated one or more of these criteria.

Because our experiments were done with display mimics and not live displaying males, we do not know whether there is a subsequent change in swimming behavior once the female locates herself above a signaling male, where she is also normally in the vicinity of sneaker males. For any given display in the field there are, on average, 3.5 additional non-signaling, sneaker males in closer proximity above a displaying male (Rivers Chapter 1). Thus for a female to be able to approach a display to within a few centimeters of the next pulse may not be sufficient for a female to ultimately locate the actual signaler. In all lab cases, when a female approached, she continued swimming diagonally through the vertical axis of the display trajectory rather than altering course to swim upward along the display path. This lack of change of swimming behavior upon reaching the display indicates that a female is either 1) relying solely on her ability to intercept the displaying male as a result of her swimming trajectory; 2) expecting to change her swimming behavior upon detecting a second stimulus (e.g. chemical trail or turbulence) produced by the male; or 3) leaving her own stimulus (e.g. a chemical trail) and expecting the male to approach and intercept her. Chemical cues are known to play important roles in many invertebrate mating systems, including crustaceans (Yen et al. 1998, Vickers 2000). Thus, we

hypothesize that luminescence may be only the primary mating signal, and a secondary signal from either the female or the male may play a role in fine-tuning of the final approach between the individuals, thus representing intersensory duetting.

Some support of a pheromone possibility comes from observations when males and females were confined in aquaria in the dark. With IR videography we have documented multiple (n >20) instances of males making dramatic shifts in swimming direction or pattern in the presence of females either swimming or stationary on the side of a tank. Many of these males approached and grasped the female and the pair then dropped toward the bottom of the tank, spinning rapidly with the male sometimes luminescing intermittently, but the female was always able to dislodge the male (sometimes after several minutes, but most within seconds), and the male would most often immediately begin to display. After each of these cases, the females were isolated, but in no case did a female produce a brood, thus indicating no insemination and hence copulation failure [n= 20]. A plausible hypothesis supported by these data, but not yet tested, is that a female approaching a display intercepts the vertical path *above* the displaying male, and she signals the male by producing a chemical trail to attract the displaying male to intercept her. The closer she is to the luminescent pulse when she intercepts the display trajectory, the more likely that only the signaler, and not sneaker males, will detect her cue.

Observing female responses to luminescent displays provides insight into what luminescent behavior we should expect to see from males. The fact that females compensate their swimming direction after nearly every pulse while not changing speed **(Table 3.1e)** suggests that it would benefit the displaying male to make as many pulses as possible. In the field, however, male-male competition is fierce, and rival males may be attempting to interfere with a displaying male to minimize the number of pulses he can produce. In laboratory observations, we have found that male display

train length (pulses per train) is inversely correlated with male density (Rivers Chapter 2), but train length determines the maximum distance a female can respond from and still intercept a male. Females swim at a mean speed of 4.52 cm/s, and the longest male train duration is about 14 seconds and about 65 cm in total length (Rivers Chapter 1). Thus, the female could be up to about 63 cm from the display train termination point and still intercept a male exhibiting the longest display. The average display in the field, though, contains 12 pulses and is 10 seconds long (Rivers Chapter 1); a female would have to be nearly 18 cm closer to the display (45 cm) in order to intercept it, a considerable decrease in effective range. Besides longer train durations, multiple consecutive display trains by a single male should also increase the likelihood of attracting and mating with a female. If a female was approaching the male's display, which stopped because of male interference, it may be able to regain the female's attention by displaying as soon as possible in the same area (Rivers Chapter 1); indeed, we see individual males display multiple times in succession in the lab in the same general location (Rivers Chapter 2).

The behavior of *V. annecohenae* females to luminescent courtship displays differ significantly from other known courtship systems. Both females and males are moving many times their own body length every second in 3-dimensional space. However, females are required to recognize and react to multiple, rapid (ca. 200ms) signals spaced about 4 cm apart and produced every 700 ms, which, by themselves, do not show the velocity or direction of the displaying male. Only by integrating multiple luminescent pulses or by having a preset swimming behavioral response that closely matches the swimming pattern of the displaying male will a female be successful in approaching and intercepting her chosen male in dark, open-sea space. The likelihood of a multimodal dialogue (luminescence for males, pheromones or other cues for females and perhaps males) adds an even higher level of complexity to this system.

Future laboratory experiments, where aspects of the display and signal are controlled, will give us the answers to these questions, and will further our knowledge of how animals, from tiny crustaceans to humans, intercept moving targets.

Methods

Female collection: Females used for the LED trials were collected (along with juveniles and males) off the south shore of Southwater Caye, Belize in small 4 cm diameter x 8 cm long PVC-pipe traps with 500μm mesh funnels at each end, and using fish muscle as bait. Virgin subadult females (5^{th} of a total of 6 instars), termed A(adult)-1 juveniles, were isolated and reared in 15cm (ca. 0.5 L) Tupperware containers (and fed Tetramin tropical fish flakes every 2 days) until each molted to adulthood (6^{th} instar). Trials using these virgin females were carried out between June-July 2005 and January-March 2006.

Male collection: A 500μm mesh cloth sweep net (25 cm diameter, 50 cm length) was used to collect male ostracods from their displays. We waited underwater until a male started the helical phase of the display (usually the third flash)(see Rivers Chapter 1), swept the net upward through the display, and then twisted close the net after each sweep. These males were maintained in isolation similarly to the females.

LED Display Mimic: The LED display that was used to mimic male ostracod displays consisted of a wand of 12 blue (460 nm) light-emitting diodes (LEDs) (E934MBD, eLED) arranged vertically at set heights to mimic the spatial patterns of the display trains of males collected in the field. The wand was located immediately behind the aquaria, and approximately 20 cm from the left side. The light output of each LED was reduced by using resistors, and all were covered with 3 sheets of 66% reduction neutral density filters to closely match the intensity of male displays. Pulse durations and interpulse intervals were controlled by an Atmel ASTK500-ND AVR

pulse microcontroller starter kit (Atmel Corporation), using an AT90S8515 8-PC microcontroller (Atmel Corporation). The microcontroller was programmed with the CodeVision AVR C Compiler program (HP InfoTech).

Recording Protocol: Females not used in each trial were maintained in their containers under a 15 watt fluorescent light in order to control for time in darkness. For each trial, 8 females were placed in a 76 cm (height) x 61 cm (width) x 15 cm (depth) clear acrylic tank filled with (70 liters) clean seawater collected on the lagoon-side of the island off the dock and not far from the display grounds. Females were left in the tank with the overhead incandescent lights on for 20 minutes, after which the lights were extinguished. We then waited for an additional 20 minutes before recording. Infrared illumination for filming was supplied from above (1cm above the waterline) by a rheostat-controlled 15-watt red frosted incandescent bulb further restricted by an infrared barrier filter. A high-sensitivity (0.00015 lux) low light 1.25cm CCD camera (Watec LCL-902K) with a 12mm aspherical low-light TV lens (Computar HG1208FCS-HSP) was fed into a Sony DCR VX-2000 miniDV camcorder, which was used in VCR mode. After 5 minutes of recording without displays, the LED display was turned on for 15 minutes. The 5 minutes of darkness and 15 minutes of displays was repeated twice more for a total trial time of 1 hour.

Analysis Protocol: We analyzed responding female swimming patterns by marking the female's position every 2 frames (66.7 ms), as well as the location and timing of the LED pulses. For analysis, pulse height was used instead of pulse number, to control for the slight variation of pulse distances during a display. Positions and distances were measured using ImageJ 1.32J image-analysis software (NIH). Interception points were calculated by finding the vector of the female 166.7 ms (5 frames) after each pulse (to allow time for swimming correction), and extrapolating a straight line to the interception point of the female above the vertical display on the y-

axis. Using the projected interception trajectory, we also calculated the angle of the female to the LED, with the interception point as the vertex **(Figure 3.2e).** Angles and distances were also calculated by using Image-J image analysis software (NIH). The swimming pattern was digitized, and Best-fit lines were calculated to determine the overall swimming slope and linearity (r^2). The linearity of swimming between pulses was calculated from the r^2 of best-fit line calculated 166.7 ms after each pulse.

To be as conservative as possible, female controls were required to be swimming at a position equivalent to the location of the females responding to the pulses but in the absence of LED pulses. Because our computer-controlled displays were constant in the experimental trials, we could then use the equivalent timing of the display to mark where the female would be if, in fact, a display mimic was occurring. Thus the protocols were identical except for the actual emission from the LED array.

Statistical Analyses: There are three main components of the female swimming pattern that were analyzed to indicate response to the male signals: 1) height of interception point above the LED, which shows trajectory; 2) the linearity of swimming between pulses, which shows consistency of the trajectory; and 3) the overall slope and linearity of swimming, which, if linear, would indicate a focused directionality. We analyzed 11 nonrandom females and 14 random females, for a total to 25 responses. All statistical analyses were performed in SAS, where we confirmed whether all assumptions of the models were met.

Interception Point Distance: Because we had up to 12 observations (=pulses) per individual, we used a backwards stepwise random coefficient mixed model to account for individual variance. Factors that might influence how far above the LED the female trajectory was aiming were 1) pulse height (which corresponds to pulse number), 2) whether the pattern was from the control or response treatment, 3) the horizontal distance of the female from the LED, 4), the vertical distance of the female

from the LED, and 5) various interactions of the above factors. Our final model to predict the intercept height distance for control females thus became:

Intercept Height Distance (cm) = 9.2789 -0.3175(Pulse Height) + 19.0064(Control Treatment) -0.6938(Pulse Height*Control Treatment) + 0.2810(Horizontal Distance [x]) -0.7480(vertical distance [y]) + 0.03820(Pulse Height *vertical distance [y]* Control Treatment)

Linearity between pulses: For the same reasons outlined above, we used a random coefficient mixed model to account for individual variance. Factors that might affect linearity between pulses were 1) the pulse number, 2) the type of treatment of the female response , and 3) any interactions between the two. The final model to predict the linearity of control females became:

Linearity (r-square) = 0.9523-0.01090(Pulse Number) - 0.1913(Control Females)

Overall Slopes and overall linearity: Since we were interested in only the overall trends of linearity and slope between treatments, we had only one observation per response, thus we did not use mixed models to control for variance. We used typical general linear models to test whether 1) treatment, 2) horizontal distance from the LED at the start of the display, 3) vertical distance from the LED at the start of the display, and 4) interactions between the above factors had any effect on the over slope and linearity of a female's swimming pattern. Neither horizontal or vertical distance nor any interaction was found to be significant, and all were dropped from the final models.

Swimming Speeds: Since males respond to another male's display by increasing their swimming speed (Rivers Chapter 2), we hypothesized that females would respond similarly. A 2-sample T-test was used to compare the mean swimming speeds of responding and control females

Potential Artifacts: In the field, the display arena of the males is a flat, fairly homogeneous open grassbed unbounded by any vertical sides. However, in our experiments females were confined tanks in the lab, which may have introduced potential artifacts to cause female responses to deviate from their natural field behavior. These effects may have been: 1) tank edge effects, which could limit or block normal female behavior to displays, and 2) the clear acrylic sides of the tank, which produce reflections of the LED display and could make it difficult to determine whether the female was responding to the primary light source or a reflection of that light. The use of a large, shallow tank helped minimize both the edge effects and the reflection issues, but did somewhat limit the height of the displays. Since we are unable to track the tiny (ca. 2mm) females in the field in the dark, we do not yet know the initial location of receptive, responding females in natural situations during a courtship display in the field. In the lab, the females in most cases tended to swim back and forth in the upper third of the tank, which may suggest that receptive females do the same in the field.

REFERENCES

Boeddeker, N. and Egelhaaf, M. 2003. Steering a virtual blowfly:simulation of visual pursuit. *Proc. R. Soc. Lond. B.* 270, 1971-1978.

Cohen, A. C. and Morin, J.G. 2003. Sexual morphology, reproduction and the evolution of bioluminescence in Ostracoda. In: *Paleontological Society Papers* [Chapter 4] 9: 37-70.

Collett, T. S. and Land, M. F. 1978. How hoverflies compute interception courses. *J. Comp. Physiol*. *A.* 125 (3): 191-204.

Gilbert, C. 1997. Visual control of cursorial prey pursuit by tiger beetles (Cicindelidae). *J. Comp. Physiol., A.* 181 (3): 217-230.

Greenfield, M.D. 1994. Synchronous and alternating choruses in insects and anurans: common mechanisms and diverse functions. *Am. Zool.* 34: 605-615.

Hastings, J.W. and Morin, J.G. 1991. Bioloumineseece. In: *Offprints from neural and integrative animal physiology*, (ed. by C. L. Prosser) New York: Wiley-Liss, Inc.

Herring, P.J. 2000. Species Abundance, Sexual Encounter and Bioluminescent Signalling in the Deep Sea. *Phil Trans R.Soc. Lond., B.* 355 (1401): 1273-1276.

Lenoir M., Musch E., Janssens M., Thiery E., and Uyttenhove, J. 1999. Intercepting moving objects during self-motion. *J. Motor Behav.* 31 (1): 55-67.

Lloyd, J.E. 1966. Studies on the pulse communication system of *Photinus* fireflies. *Misc. Pub. Mus. Zool*. *U. Mich.* 130: 4-95.

Lloyd. J.E. and Wing, S.R. 1983. Nocturnal aerial predation of fireflies by light-seeking fireflies. *Science.* 222: 634-635.

Morin, J.G. 1986. 'Firefleas' of the sea: luminescent signaling in ostracode crustaceans. *Fla. Entomol*. 69: 105-121.

Morin, J.G. and Cohen, A.C. 1991. Bioluminescent Displays, Courtship, and Reproduction in Ostracodes. In: R. Bauer and J. Martin, eds., *Crustacean Sexual Biology.* pp. 1-16. New York: Columbia Univ. Press.

Olberg, R. M., Worthington, A. H. and Venator, K. R. 2000. Prey pursuit and interception in dragonflies. *J. Comp. Physiol*. *A.* 186: 155-162.

Port, N.L., Lee, D., Dassonville, P., and Georgopoulos, A.P. 1997. Manual interception of moving targets .1. Performance and movement initiation. *Exp. Brain Res.* 116 (3): 406-420.

Torres and Morin, *in press.*

Vickers, N.J. 2000. Mechanisms of animal navigation in odor plumes. *Biol. Bull.* 198(2): 203-212.

Wagner, W.E. Jr, and Reiser, M.G. 2000. The importance of calling song and courtship song in female mate choice in the variable field cricket. *Anim. Behav*. 59: 1219-1226.

Yen, J., Weissburg, M.J., and Doall, M.H. 1998. The fluid physics of signal perception by mate-tracking copepods. *Phil. Trans.R. Soc. Lond., B*. 353: 787-804.

CHAPTER 4

LUMINESCENCE IN CYPRIDINID OSTRACODS: LIGHT BUDGETS AND BEHAVIORAL FUNCTIONS

Introduction

Bioluminescence has evolved for many different functions in a wide variety of organisms ranging from bacteria to fish, and in terrestrial and aquatic, especially marine, environments (Morin 1983; Hastings and Morin 1991). Along with being used in sexual selection, luminescence is used to attract (as a lure) or illuminate prey, for counter- or disruptive illumination (to break up the silhouette from upward-looking visual predators in marine systems), as an advertisement of distastefulness (aposematism), and as a way to startle predators or attract a predator of the attacker (the burglar alarm effect) (Burkenroad 1943; Morin 1983, 1986). Although exhibiting one type of luminescent behavior has been described in a wide variety of organisms, there are fewer known systems where a single organism exhibits multiple behavioral uses for light production. Midwater fishes may use luminescence as a lure, while at the same time using other light for counterillumination (see Morin 1983). Adult fireflies utilize light in species-specific flashing patterns, but also flash when being attacked by predators (Lloyd 1983). The flashlight fish *Photoblepharon palpebratus,* found in the Red Sea, apparently uses luminescence to see and attract prey, to confuse and avoid predators, and for aggregation and likely courtship (Morin et al 1975). All of these cases involve intracellular or symbiont luminescence, where the light emanates from within the body or cell, not extracellularly.

The substrate that the majority of luminescent organisms in the marine environment use in light production is either coelenterazine or cypridinid luciferin, which are both tripeptides with imidazolopyrazine cores. These strong antioxidants are hypothesized to have first evolved for protection against free radicals, and later were

co-opted for luminescence (Rees et. al 1998). Neither cypridinid-type luciferin nor coelenterazine are restricted to a single phylum (see Hastings and Morin 1991; Shimomura 2006). Luminescence involving cypridinid luciferin is found in cypridinid ostracods and some species of fish, while luminescence utilizing coelenterazine has been found in no less than seven separate phyla (Haddock and Case 1994). In both of these cases, some of the organisms that utilize the luciferin are not capable of producing this substrate on their own; rather, they obtain it from their prey, but they are able to synthesize the luciferase enzyme (Tsuji et al. 1972; Haddock et al. 2001). It is possible that these molecules are synthesized in only one or a few phyla, indicating a potentially complex transmission of chemicals active substrates through predator/prey interactions. Myodocopid ostracods, the focus of our study, appear to be able to synthesize both their own cypridinid luciferin and luciferase in special secretory cells in the upper lip (Huvard 1993a).

Myodocopid ostracod luminescence has a long and extensive laboratory history of study. First described by E. Newton Harvey in the 1920's, much work has been done on the kinetics, molecular structure, uptake of luminescent precursors by other animals, and biosynthesis of the luciferin molecule (for review see Harvey 1952; Herring 1978; Shimomura 2006), as well as the mechanisms of secretion of luminescence into the water column (Huvard 1993a). The reaction shows simple first-order kinetics where output (light) is directly proportional to the amount of enzyme (luciferase) present. This relationship makes it relatively easy to calculate the luciferin/luciferase ratios. The color is bright blue, with a peak at 473 nm (Huvard 1993b), which is near the peak wavelengths least attenuated in seawater (Jerlov 1968).

The majority of studies have focused on systematics and evolution and the physical and chemical aspects of ostracod luminescence, but there has been less focus on behavioral aspects. Apparently all species of light emitting myodocopid ostracods

luminesce brightly in response to predation attempts (Morin 1986; Morin and Cohen 1991) and these are found in nearly every world ocean (Harvey 1952; Korniker 1984; Cohen and Morin 2003). Some species have been reported to respond to a human-induced light flash (with a flashlight) by releasing luminescence of their own; the reason for this behavior is still unknown (Tsuji et al. 1970). The control of the secretion of the luminescence is intricate (Huvard 1993a), but, because it is extracellular, it does not have as complex regulation as in firefly displays and other internally regulated systems. However, there is a large monophyletic clade of Caribbean ostracods that not only utilize light for defense, but also for courtship (Morin 1986; Morin and Cohen 1991; Rivers Chapter 1,2). Each species has its own unique display pattern. There is a spectacular variety of these pattern that range in direction from vertical up or down, lateral, or diagonal; in pulse number from few to many pulses per display train; and in pulse durations that range from less than 100 ms to several seconds. From here on, our use of the term 'courtship display' includes the entire courtship display train and all pulses within. All of these displays, as is the antipredation response, are extracellular: the luminescent molecules are secreted through nozzles in the upper lip of the ostracods, along with mucus which is hypothesized to bind the chemicals together (Huvard 1993a). In these ostracods, all larval stages and both sexes are able to release luminescence in response to predation, but only males utilize light in the complex, species-specific patterns to attract a female in specific microhabitats (Morin 1986; Morin and Cohen 1991). There is no luminescent dialogue between males and females during courtship; rather, the female silently approaches the display, and with the possible secondary sensory cues, intercepts the displaying male (Rivers Chapter 3).

The focus of the present paper is to describe and quantify the luminescence of both courtship and antipredatory displays of *Vargula annecohenae,* an ostracod that

produces courtship displays in high numbers above the grassbeds of Belize (Torres and Morin, *in press*), and to compare the total luminescent output of these two behaviors to each other as well as to the total light available in an individual. We show that single courtship displays, while bright, repetitive and abundant, do not significantly deplete luminescent reserves, while each antipredatory display, which produces an order of magnitude more luminescence than a typical courtship display, can deplete the stores if triggered more than about four times in succession or per night.

We provide the first description comparing the kinetics of the two markedly different extracellular luminescent behaviors from a single organism. We also discuss the possibility that this aposematism could possibly indirectly benefit non-luminescent planktonic organisms by suppressing predation.

Methods

Collection: Ostracods were collected in baited traps placed on the sand surface in shallow grassbeds or via sweep nets during the displays (for details see Rivers, Chapter 1).

Photomultiplier Tube [PMT] setup and analysis: Light-intensity was recorded with an RCA 931-A photomultiplier tube (PMT), covered by an Andover 039FG11-50 3mm Infrared (IR) barrier filter, at a distance of 76 cm from the aquaria. The PMT was powered by an Emco Ca12N High voltage converter (set to 1000v). The PMT output was connected to a Dataq DI-158U analog data acquisition (DAQ) device which recorded 240 data points per second on a Dell laptop, using the program WinDAQ. We quantified different displays in relative terms and not in terms of total photons or energy. We report all values relative to the mean output of a courtship display, which is the most consistent and least variable of the various emissions.

Because different outputs differ by over two orders of magnitude, based on experience, the gain was preadjusted to maximize the resolution without saturation. To analyze the waveforms and relative light output, the digital data were converted to a spreadsheet and exported into excel and the program Origin.

Courtship display trains: Since ostracod luminescence is a simple first-order reaction (Harvey 1952), rise and decay rates of the luminescent pulses of *V. annecohenae* are exponential, and the waveforms vary with temperature. We used photometric (PMT) recordings to determine waveform and total light data (as above). Our experimental setup followed the same methods as used in previous studies to observe individual behavior (Rivers Chapter 1, 2), with the PMT replacing the video camera. As before, we used 5 males together per trial. The courtship display data from the PMT were converted to a spreadsheet and imported into the program Origin, where the exponential rise and decay rates of individual pulses were calculated using the 1st order exponential equation $y = Asymptote + Amplitude*(e)^{-x/decay\ constant}$. We compared the decay rate constants of the first pulse of 33 courtship trains during 4 trials at 2 different temperatures (2 trials in February 2006 at 26 ^{o}C, and two in July 2005 at 30 ^{o}C). We further analyzed multiple pulses per train for as long as they were clean peaks with little noise for accurate results (at 26 ^{o}C N = 22 display trains with 36 pulses analyzed; at 30 ^{o}C N= 11 display trains with 29 pulses analyzed) for a total of 65 pulses. Peaks with high noise to signal ratios (due to low intensity) were not included in our analyses. To determine the effect of temperature or pulse number on the decay rate constant, we log-transformed the data to satisfy the equal variance assumption, and used a random effects mixed model to correct for multiple data points from individual displays (SAS 9.1). We did not have enough degrees of freedom to compare the rise and decay rates between different displays. The rise and decay rates of each peak were also compared to each other using a paired-t test (SAS 9.1).

The total light emitted per pulse and per courtship display train was also calculated from the PMT data. Data were imported into Origin and the area under the peaks, which corresponds to total light emitted (in volts), was calculated. The luminescence output per total courtship display was compared to the luminescence output during both antipredation displays and when individuals were crushed to produce maximum total light output from an individual (see below).

Antipredation displays: Six dusky cardinalfish (*Phaeoptyx pigmentaria)* were collected in a 500 μm mesh net while snorkeling at night at a patch reef off the southwest tip of Southwater Caye, Belize. To prevent injury, each fish, once netted, was immediately transferred to a 4.5-liter seawater-filled Ziploc plastic bag. In the lab they were kept in opaque, blue 20-liter buckets each with 10 liters of fresh sea water and an opaque 15cm diameter x 15 cm length PVC pipe for shelter, and the seawater was replaced daily. After being kept without food for two days, we placed two individuals (to maximize predation attempts in a given time period) in a 15(w) x 16(l) x 30(h) cm clear acrylic tank, filled to a depth of 10 cm (=2.4 liters). The seawater was collected off the nearby dock during the day (to minimize potential biases from other luminescent sources). After it became dark, the lights were turned off, and the fish were allowed to acclimate for an hour. Six *V. annecohenae* ostracods were then placed in the tank in the dark, and we recorded the light output (with both the PMT for waveform data and the video camera for fish behavior) that resulted from predatory attacks on the ostracods by the fish. The maximum intensities and total light produced (area) from 16 antipredation episodes were analyzed per antipredatory display as described above. In addition, the decay rates from 53 clean parts of antipredation displays were calculated using the same methods as above; we did not calculate the rise rate because luminescence occurred within the buccal cavity of the fish predator before being pumped through the gills. The decay rate constants were log-transformed

to satisfy the assumption of equal variance. To determine the differences between the decay rate constants of antipredation displays (n = 53) and courtship displays (n= 36) at the same temperature (26 ^{o}C), as well as to determine whether the pulse (or peak) number had any effect on the decay rate constant, we once again used a random-effects mixed model in SAS. The fishes were returned to the field immediately after the trials.

Total Potential Luminescence: Seven male and six female ostracods were collected via traps the night before they were transported back to Cornell University, where we determined the total available luminescence per ostracod. The day after returning to our Cornell lab (2 days after collection), individuals were placed in a 5ml tissue grinder tube in 0.5 ml artificial seawater. In a darkened room, 0.5 ml of *fresh water* was added, which induced luminescence, and then the individual was crushed and ground incrementally. This procedure allowed us to induce the total luminescence from an individual without exceeding the least sensitive setting of our recording equipment. The data were imported into Origin and the area (total light) per individual was calculated. Since the crushed individuals' luminescence is not under the ostracods' behavioral control, we only compared these results for total light output and not kinetics. We then compared this total light emitted to light output from both antipredation and courtship displays (see below).

Comparisons of the types of luminescence: To determine whether we could pool the total light emitted from males and females, we log transformed the areas and ran a general linear model (GLM) in SAS. There was no significant difference between the males and females ($f = 0.11$, $p = 0.7448$), so we pooled the results. The light emitted from courtship displays, antipredation displays, and the pooled total body stores of *V. annecohenae* were log- transformed to obtain equal variance between treatments, and a general linear model (GLM) in SAS was used to compare treatments.

Daytime palatability experiments: Eight juvenile (ca. 2 cm) Sergeant Major fish (*Abudefduf saxatilis*) and three juvenile (ca. 3cm) Beaugregory fish (*Stegastes leucostictus)* were collected in shallow areas surrounding Southwater Caye during the day, using a 20-cm diameter, 20 cm-long 500 μm mesh sweep net (December 2001) and placed in clean, fresh seawater in a large 34 liter cooler, and kept without food for 2 days. These fishes were then placed in individual 15cm (1 liter) Ziploc bowls with one of four potential food sources, and their behavior was recorded under ambient daylight conditions. For each fish, four food source treatments in randomly-selected order were added to the containers and the behavior of each was recorded for five minutes. The treatments were: 1) live *V. annecohenae,* 2) *V. annecohenae* killed in hot seawater (>85 ^{o}C), 3) a live nonluminescent myodocopid ostracod *Skogsbergia (cf. lerneri)*, and 4) recently thawed frozen fish muscle (ca 15 mm^3) .

Nocturnal feeding experiments

In addition to the video recordings during the antipredation experiments above, at night in December 2001 we collected 3 dusky cardinalfish (*Phaeoptyx pigmentaria)*, from the patch reef off the southern edge of Southwater Caye, and 3 silverside atherinid fish (*Hypoatherina harringtonensis*) (see methods above). After being kept without food for 2 days, the fishes were individually placed in small 15 cm (1 liter) plastic Ziploc aquaria, and 2 luminescent ostracods and 2 nonluminescent *Skogsbergia* ostracods were added (one at a time in random order) in each trial. A video recording was made using the Sony DCR VX-2000 camera and dim red light (from a filtered flashlight).

Results

Luminescent budgets- Light output comparisons: Our data show that, while courtship display trains are highly visible and repeated over and over on any given

night in the field, they constitute only a small fraction of the luminescence that are spewed out when an ostracod is attacked by a predator (**Figure 4.1).** Furthermore, these antipredatory displays themselves represent only a fraction of the light available from the total stores available within an individual. There were significant differences in total luminescence between courtship displays, antipredation displays, and crushed individual luminescence (DF 36, F = 77.56, p <0.0001) **(Table 4.1a,b, Figure 4.2)**. Crushed individuals released such large quantities of luminescence in the tissue grinder that the light was visible to the dark adapted eye for at least 3 minutes.

The average antipredation episode comprised only about one tenth of the total luminescent stores found in crushed individuals, but was about 50 times the amount of luminescence from a typical courtship display train (of 9 pulses) from our laboratory results **(Table 4.1a,b, Figure 4.2)**. Thus, a single courtship train of 9 pulses represents only about one five-hundredth (0.2%) of the total stores. Previous studies (Rivers Chapter 1) have shown there was a mean of 12, and a maximum of 19, pulses per display train in both laboratory and field and the final 10 pulses were nearly equal in intensity and waveform. Thus, we extrapolated our data to predict the luminescent percentages found in both the mean and maximum display trains **(Table 4.2)**. A courtship display consisting of 12 pulses would be 2.1% of the mean antipredation episode and 0,21% of the mean total luminescence stores, while a 19-pulse courtship display would be 2.3% of the mean antipredation episode and 0.22% of the mean total luminescence stores. The brightest pulse of an average antipredation display was 13 times brighter in intensity than the brightest pulse of an average courtship display train, and showed much more variation between peak intensities (SE = 1.11 for antipredation, 0.0008 in displays) **(Table 4.1a,c, Figure 4.3)**. During a predation attempt, each ostracod released copious quantities of luminescence that appeared as pulses, which coincided with the pumping frequency of the fishes gills. As a result of

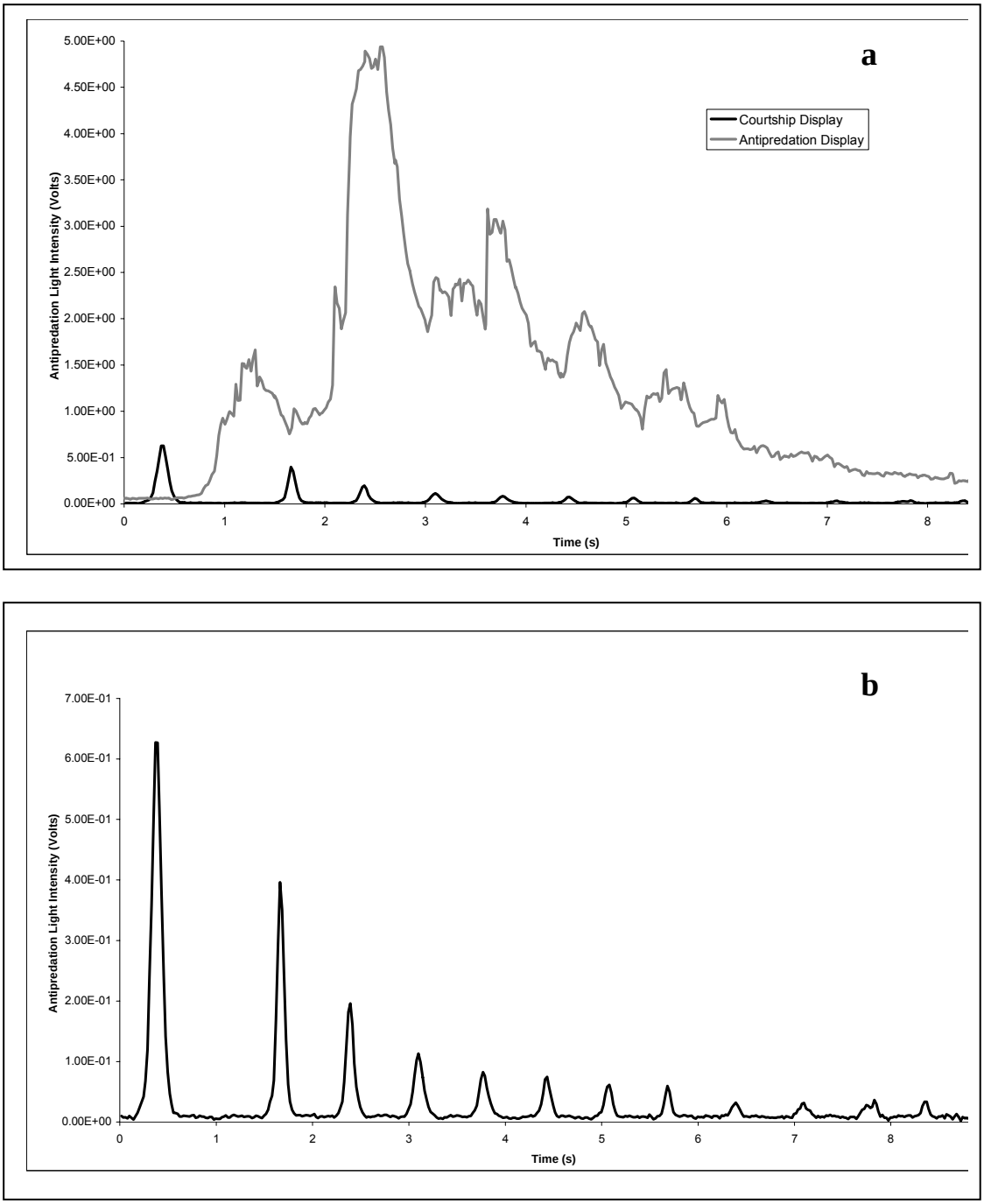

Figure 4.1. a. Comparison of Antipredation (gray) and courtship display (black) luminescence output in equal relative units. The variable peaks in the antipredation luminescence are due to the fish predator pumping its gills and ejecting the luminescence from its mouth in addition to the ostracod releasing luminescence in pulses as it is disturbed. **b.** The courtship display is the same as seen in **4.1a**, but at higher resolution.

Table 4.1. **a)** Total light and peak intensity values for courtship display behavior, antipredation behavior, and total luminescence, and percentages of mean and maximum values between the **b)** areas and **c)** the intensities of antipredation and display behaviors. Values correspond to row over column percentages. For example, in **b)** the mean display area is 1.98% of the mean antipredation area, and 0.20% of the mean total luminescence found in a single individual.

a. Total light and peak intensity values of luminescence (relative units [Volts])

	Area (=total light)		Peak Intensity		N
	Mean	**Max**	**Mean**	**Max**	
Antipredation	3.41 +/- 1.11	15.90	2.45 +/- 0.40	4.88	16
Courtship display	0.067 +/- .008	0.12	0.191 +/- 0.034	0.60	14
1st Pulse	0.028 +/- .005				14
Helical Pulse	0.0033 +/- .0002				14
Total animal	34.91+/- 4.07	62.68	NA	NA	13

b. Light Output Comparisons

	Courtship		***1st Pulse***	***Helical Pulse***	**Antipredation**	
	Mean	**Max**	**Mean**	**Mean**	**Mean**	**Max**
Courtship						
Mean			41.86%	4.91%		
Max			23.30%	2.73%		
Mean 1st Pulse				11.73%		
Antipredation						
Mean	1.98%	3.57%	0.83%	0.10%		
Max	0.42%	0.76%	0.18%	0.02%		
Total Stores						
Mean	0.20%	0.36%	0.08%	0.01%	10.14%	25.36%
Max	0.11%	0.19%	0.04%	0.005%	5.44%	10.14%

c. Peak Intensity Comparisons

Courtship		
Antipredation	**Mean**	**Max**
Mean	7.77%	4.96%
Max	3.90%	12.23%

Table 4.2: Characteristics of courtship display luminescence output during the stationary (grey) and helical (white) phase.

Pulse #	Mean % of Pulse 1 [N]	% of total found in Stationary Phase	% total found in Helical Phase
1	100% [14]	100%	0%
2	48.55 +/- 4.89% [14]	100%	0%
3	29.62 +/- 3.38% [14]	100%	0%
4	21.68 +/- 2.20% [14]	89.15%	10.95 %
5	17.13 +/- 1.55% [14]	82.11%	17.89%
6	15.03 +/- 1.42% [14]	76.79%	23.21%
7	13.39 +/- 1.41% [12]	72.60%	27.40%
8	8.35 +/- 1.09% [4]	70.21%	29.79%
9	9.41 +/- 1.41% [4]	67.70%	32.30%
12*	8.0%Φ	62.04%	37.96%
19**	8.0%Φ	51.92%	48.08%

* Mean number of pulses per display in both the laboratory and the field (Rivers Chapter 1)

**Max number of pulses per display in both the laboratory and the field (Rivers Chapter 1)

Φ Since we were unable to accurately measure the area of pulses past the 9th pulse, we set the percentage of the 1st pulse to be 8% for pulses 10-19

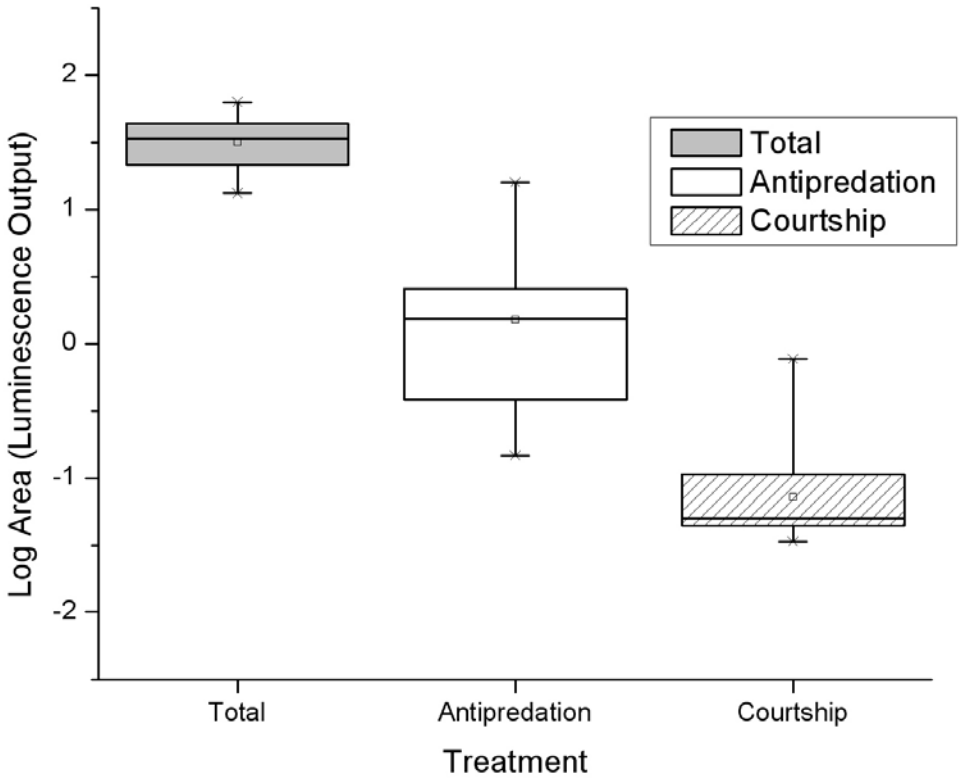

Figure 4.2. Boxplot comparisons of the total luminescence found per ostracod and the outputs for antipredation and courtship display behaviors. Data were log transformed for analysis. The box comprises of the middle 50% of the observations, the horizontal line is the median, and the circle is the mean. The arms extend to 95% of the range.

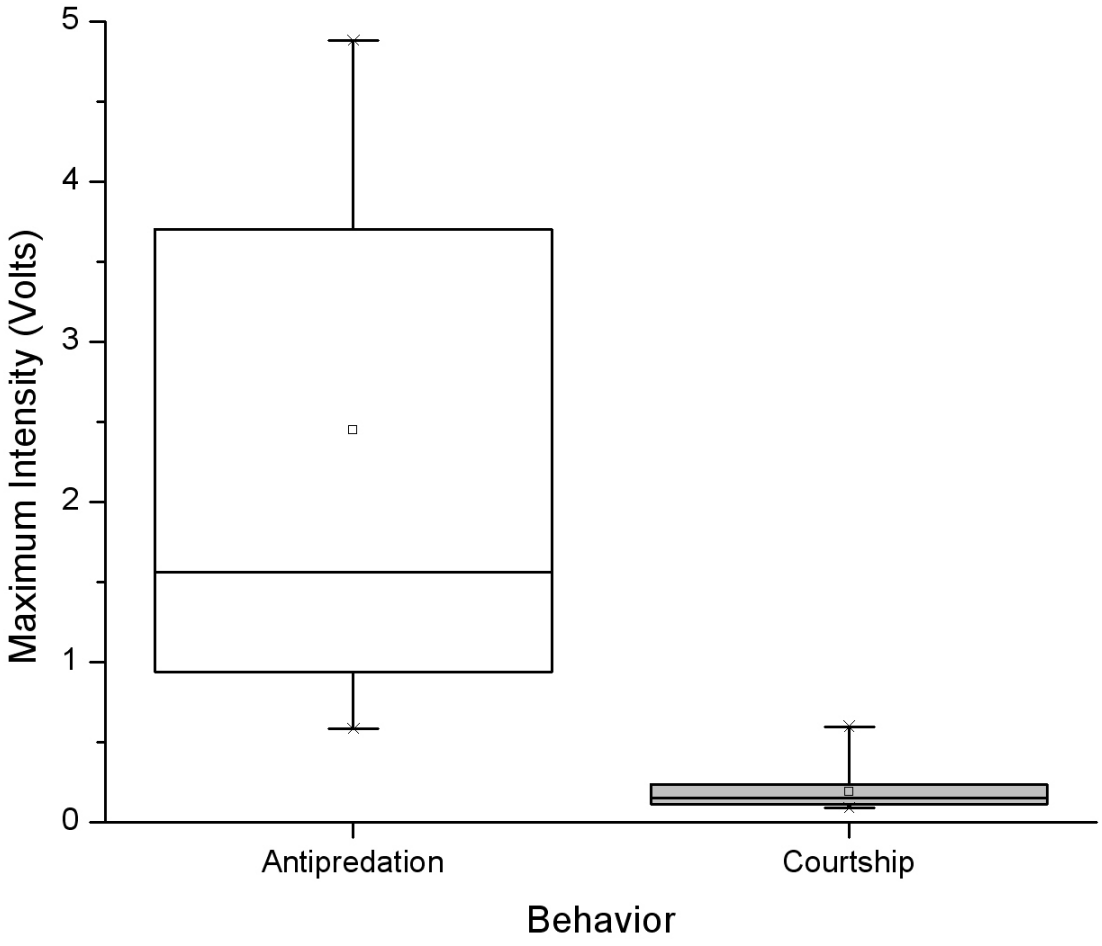

Figure 4.3. Maximum intensity comparisons between antipredation and courtship display behaviors. Data were log-transformed to satisfy the assumptions for linear regression analysis; these raw data show the wide variation in luminescence intensities during an antipredation episode vs. during courtship displays.

the gill pulsations we do not know if the ostracods release a continuous stream of luminescence or not **(Figure 4.4).** As shown in a representative antipredatory display **(Figure 4.1)**, the pulses of light rise very quickly and peak within about 3-4 sec and subsequently gradually diminish in intensity over the next several seconds. At about this point in most trials the ostracod was expectorated from the buccal cavity of the fish and, while still glowing weakly, would usually swim rapidly away, apparently unharmed **(Figure 4.4)**.

There are two distinct phases of courtship displays which differ in their luminescence output (Rivers Chapter 1). The first (stationary) phase contains about 3-4 brighter, longer-duration pulses followed by a second (helical) phase consisting of up to 16 dimmer, more uniform pulses (Rivers Chapter 1). The mean total luminescent output of the courtship display trains in our laboratory experiments (n = 9 pulses) uses only about 0.2% of the total luminescence stores found in an ostracod **(Table 4.1b)**. Within a train, 42% of the total train output (or 0.08% of the total stores) occurs in the first pulse, while each average helical pulse contained only about 5% of the total train output (or 0.01% of the total stores)(**Table 4.1b).** We could only distinguish a maximum of 9 pulses per courtship display from our PMT recordings either because of spatial constraints of our tanks and/or the pulses became indistinguishable from the PMT noise**.** In these 9-pulse display trains, the first 3 pulses of the stationary phase contained 68% of the overall luminescence released in the train (**Table 4.2)**. The stationary phase in a display extrapolated to 12 pulses would utilize would contain 62% of the luminescence found in the display train, and 0.21% of the maximum total luminescence stores found. When extrapolated to 19 pulses for a courtship display train, the stationary phase would contain 52% of the luminescence of the train, and utilize 0.22% of the maximum total stores.

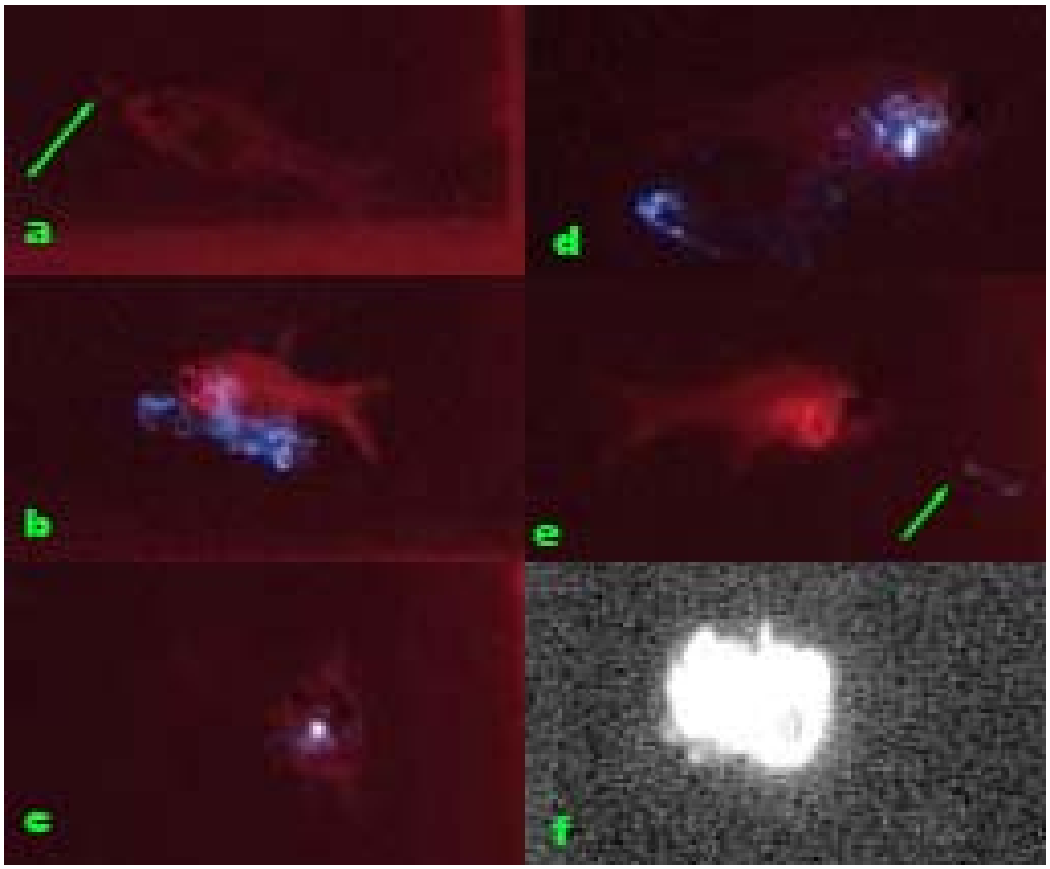

Figure 4.4. Attempted predation on *Vargula annecohenae* by a dusky cardinalfish, *Phaeoptyx pigmentaria*. **a-d** are still images taken from a video with a light source from a dim red flashlight but no light amplification, and the **a.** The arrow points to the 2mm ostracod, and shows the cardinal fish approaching. **b.** Almost immediately after being taken into the fish's mouth, the ostracod releases copious amounts of luminescence, which is being pumped from the gill opercles. **c.** Even when the fish is not pumping luminescence through its opercles, some luminescence is apparent within buccal cavity **d.** Once the fish pumps its gills, more luminescence is released into the water. **e.** The fish expectorates the ostracod (arrow); the trail to the bottom right of the ostracod is luminescence left behind as it swims away apparently unharmed. **f**. A low-light CCD still image of another antipredation episode at its brightest (the cardinal fish is in the center of the luminescent spot).

Exponential rise and decay constants of courtship displays were found to be quite constant. There were no significant differences between exponential rise and decay constants of individual display pulses (DF= 64, t= -0.09969, p = 0.92090) **(Figure 4.5)**. However, there was not sufficient power in our analyses to determine whether the rise and decay rate constants of *individual males* differ. As expected for simple 1st order reactions, temperature had a significant effect on the decay rate constant for displays, with faster decay rates in warmer temperatures (DF 2.36, F 61.45, p = 0.0097). However, there was no significant differences between pulse numbers within the same train (DF 63.7, F 0.32, p = 0.5735), indicating the same control behavior of luminescence across pulses. At 26^{o}C, the mean decay constant for pulse 1 was 0.068 (+/-0 .004), and for pulse 2, 0.070 (+/-0.004), while at 30^{o}C the mean decay constant for pulse 1 was 0.030 (+/- 0 .003), and for pulse 2, 0.023 (+/- 0.003).

Only decay constants were analyzed during antipredation displays due to the initial luminescence being obscured in the mouth of the fish. Comparing courtship and antipredation display decay rates at 26 ^{o}C, we found that neither the courtship pulse number (DF 79.4, F= 1.97, p = 0.1644) nor the interaction between treatment and courtship pulse number (DF 79.4, F = 3.62, p = 0.0608) had any significant effect on decay rate. However, we did find large differences in the rate of light decay between antipredation and courtship displays. While both antipredation and courtship displays pulses decay exponentially, the mean log-corrected decay constant of courtship display luminescence was significantly lower than that of antipredation (DF =89.3, F=9.39 p<.0029). Antipredation display luminescence decreased about four times slower than of courtship display luminescence (mean decay constant of an antipredation episode = 0.305 +/-0.044; mean display constant = 0.068 +/- 0.004) (**Figure 4.5)**.

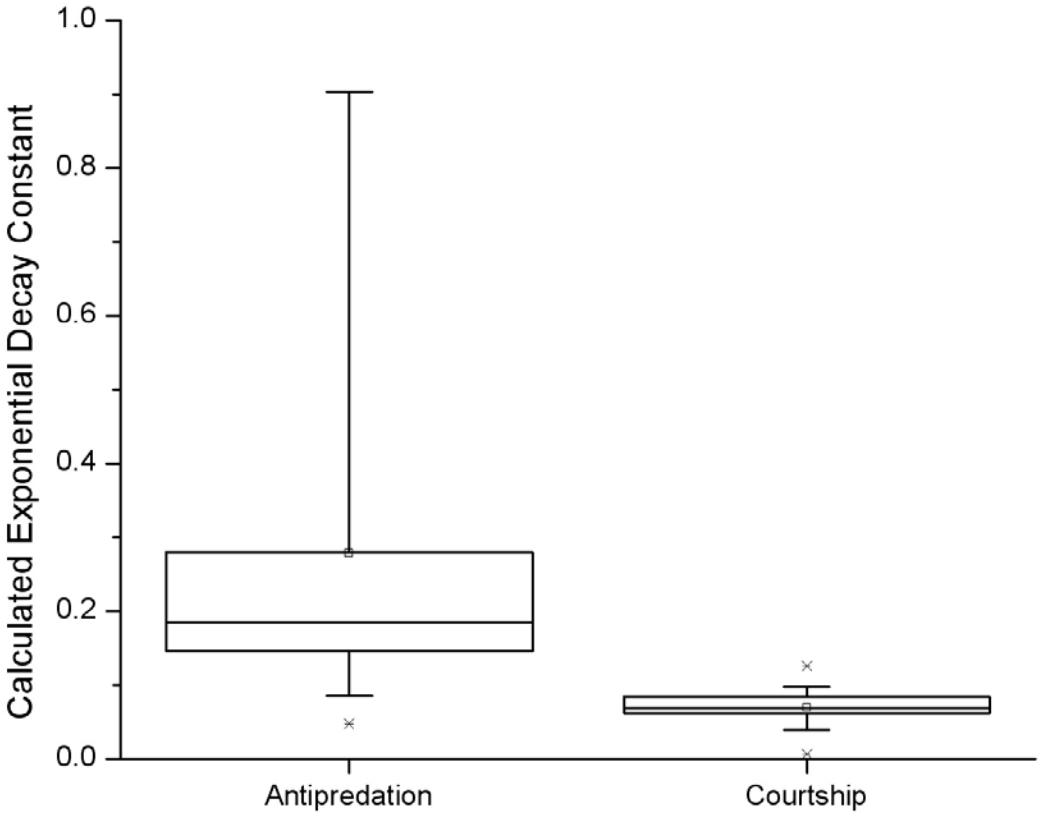

Figure 4.5. Decay constant comparisons between antipredation and courtship luminescence (for analysis, the data were transformed to meet the assumptions for regression). The higher variance in antipredation behavior could be a combination of the concentration of molecules being diluted due to increased area of luminescence after being pumped through the fish's gills with no modification of chemical ratios by the ostracod or male ostracods may exhibit a fine control over their luminescence ratios for displays, but not for antipredation.

Feeding experiments: Feeding experiments, either by day or night, resulted in the ostracod *V. annecohenae* always being expectorated by all fish species, in most cases alive, and the ostracod swam away rapidly. During a five-minute trial, there were approximately 3-4 predation attempts on *V. annecohenae,* each of which resulted in expectoration. A fish would take an ostracod into its buccal cavity, sometimes retaining it in for a number of seconds without swallowing, before releasing it **(Figure 4.4)**. In contrast, all heat-killed *V. annecohenae,* nonluminescent *Skogsbergia,* and fish muscle were readily eaten when offered to the fish.

Discussion

Luminescent Budgets: Male *Vargula annecohenae* are capable of secreting an astounding amount of extracellular luminescence from their stores. Starting with the assumption that an ostracod has a full store of luminescent compounds (cypridinid luciferin and luciferase and mucus), all of which are available to be secreted, then a typical male has the potential to produce nearly 500 average courtship displays trains consisting of 12 pulses, 10 average antipredation displays, or 4 maximum antipredation displays before depleting the luminescent potential it has stored at a given time. Within a single typical courtship display train, the first pulse contains 42% of the total luminescence of the train, while a typical pulse in the helical phase contains only about 5% of the output of a total courtship display. We use these calculations in determining the nightly luminescent budget and luminescent compound regeneration, and in considering the behavioral strategies of males participating in courtship and antipredation displays.

Courtship displays in a square-meter seagrass bed area in the field occur at an average of about once every 15 seconds (7 displays/minute), and it is highly unlikely that a single male is responsible for all of these (Rivers Chapter 1). When tracking

individual male behavior in the laboratory (Rivers Chapter 1), we found the highest courtship frequency for a single male to be about 1.5 displays per minute (unpublished). Extrapolating this individual courtship display frequency to encompass an entire hour courtship period gives the male a maximum of about 90 courtship displays per night. This number of courtship displays per night for a single male is most likely somewhat higher than the frequency of a typical displaying male, as in no case in the lab has one male been shown to display consistently over that length of time. Rather, a male will display several times, and then sneak or entrain on other courtship displays (Rivers Chapter 2). In addition, we have also seen activity stop completely for a period matter of some minutes (at least 5) before commencing again in both the lab and the field (per. obs.). However, this number of 90 courtship displays per night is a reasonable high-end maximum frequency for our estimations. Since a courtship display train of 12 pulses (the mean in both the lab and the field [Rivers Chapter 1]) uses only about 0.2% of the total stores available, a male who produces 90 courtship displays per night would consume about 19% of its total stores per night; thus, it would take over 5 nights to completely exhaust his existing stores and, therefore. it must be capable of synthesizing this amount in a 24-hour period.

Because we had collected males via sweep nets during courtship while they were luminescing and which induced and antipredatory kind of response when they were in the net, and females from traps, where they did not often produce antipredatory responses, the fact that there were no significant differences between adult males and adult females in total luminescence stores only two days after collection gives further evidence that ostracods are able to replenish their luminescent compounds, most likely every night. In *Vargula* ostracods, luminescent compounds are synthesized in a luminescent organ made up of long secretory or exocrine cells, with each one extending the entire length of the light organ (ca. 500 μm) and

terminating at nozzles on the upper lip (Huvard 1993a). These cells have three separate areas: 1) the basal synthesis region, which makes up 6% of the length of the secretory cells, and the remaining 94%, which is comprised of 2) the transitional region, where the secretory vesicles containing the compounds are being transported to 3) the storage region which terminates at the nozzles on the upper lip (Huvard 1993a). How much of the total luminescence is available for use from the transitional and storage regions, and how much is unavailable for immediate use is still unknown. Based on antipredation behavior observations, we know that individuals are able to utilize at least 25% of their total stores at one time, which gives us a low-end estimate. If this is close to the *maximum* amount from storage readily available to males, and if males vary significantly in their rates of synthesis of luminescent compounds (due to intrinsic or energy reserve differences), there may be significant variation in the frequencies or intensities of courtship displays males are able to exhibit each night. Because luminescence is important in *V. annecohenae* courtship, variations in display ability may affect variation in mating success for males.

If two males had equal courtship display frequencies but varied significantly in the intensity of each display, then the rate of regeneration for full luminescence recovery would necessarily vary. In earlier studies we discovered that, among individual males, the brightest pulse of the brightest courtship display was 85% brighter than the brightest pulse of the dimmest luminescent courtship display train during laboratory trials, and 50% brighter than the brightest pulse of a mean courtship display train (Rivers Chapter 1). Therefore, some males are expending much more energy per display than others, and may release more luminescent compounds per night than others, thus requiring a higher rate of synthesis to replenish its stores. However, we also know there are differences in display frequencies between males (Rivers Chapter 2). If males with brighter courtship displays have a lower frequency

of displays per night, there may be two separate behavioral strategies utilized by males with the *same* luminescent budget. Males could invest more heavily in a brighter display but at a lower frequency over the night, hoping to attract a female by being much brighter than other displays; or they could display more dimly but more often, giving a receptive female more chances to notice them.

From an examination of the differential allocation of luminescence within a single courtship display train, we can make predictions about the energetic costliness of different potential courtship display strategies of equal pulse intensities. In an analysis of the light budgets, 50-60% of all the light produced in a train occurs during the initial stationary phase (first 3-4 pulses) while the succeeding 9-16 pulses of the helical phase contain less than half the light. However, it is this last half that is crucial for aligning receptive females to the signaling male (Rivers Chapter 3). Adding successive pulses in the helical phase, since they are all quite similar and represent only about 5% of a typical train, does not represent much of an energetic drain on the male (less than a 0.01% decrease of a male's total stores by increasing the train length from 12 to 19 pulses). Therefore, in addition to providing an approaching female more cues for successful interception and copulation (Rivers Chapter 3), it would seem to be more energetically efficient to have fewer, longer courtship display trains rather than more, shorter trains. However, if a female is easily distracted by the brighter pulses in the stationary phase of a nearby competing courtship display, although more energetically costly and providing fewer interception targets to the female, the original male may be more successful at regaining her attention (with the much brighter luminescence of the stationary phase) by abandoning his current display and starting again. Both of these competing factors may be acting at the same time upon displaying males in the field; the most successful courtship display train length, therefore, may be

a tradeoff with the average closer to the mean of 12 pulses that we see in the field than trains considerably longer or shorter.

The luminescent budget of displaying males discussed above assumes that luminescence is not being utilized for any other purpose such as antipredation. However, a single antipredation episode may deplete at least 25% of the luminescent potential with each episode (**Table 4.1)**; this may even be an underestimation of total light emitted because the fish predator may be absorbing some of the luminescence prior to reaching our detector. This depletion of at least a quarter of its stores could severely lower a male's ability to produce courtship displays in order to be sexually competitive. Antipredation events observed in the field are rare. While we observed literally thousands of courtship displays between dusk the end of each display period, on any given night we almost never observed even one antipredatory event in the same area. When antipredatory displays are observed, they almost always occur earlier in twilight when it is still somewhat light and the courtship displays have not commenced (Morin 1986; pers. obs.). Even so, based on our comparisons between luminescent reserves and antipredatory displays, a single ostracod contains enough components for up to about 4 major antipredation displays before requiring actual synthesis of new compounds, a rate of attacks we have never observed, nor would expect to observe, in the field.

Kinetics: The kinetics of the ostracod luciferin-luciferase reaction are simple first-order chemical reactions; the decay rate is logarithmic and depends directly on the enzyme concentration (see Harvey 1952; Herring 1978; Shimomura 2006). However, the majority of the kinetic work has been done on extracts in laboratory settings with known ratios of enzyme and substrate, as well as a known volume and no extraneous compounds. Trying to determine enzyme-substrate ratios in a water column, from ostracods that are controlling the release of luminescent chemicals as

well as other nonluminescent compounds, makes the determination of the exact kinetic interactions much more difficult.

Although we do not know the actual ratio of luciferin to luciferase in *V. annecohenae,* the lack of variation between pulses during courtship display luminescence indicates fine control over the chemical ratios by the ostracod. The complex structure of the light organ and surrounding muscles make this control possible (Huvard 1993a). There are 3 distinct separate portions on the lip in which nozzles are found, and from which the different compounds of luciferin (the tripeptide), luciferase (the enzyme), and mucus are separately secreted. The luciferase is secreted from the center row of nozzles, the luciferase is secreted from rows of lateral nozzles, and mucus is secreted from the outermost row of nozzles and tusks (Huvard 1993a).The method of secretion of the mucus and luminescent compounds into the water column controlled by the action of 3 different types of muscles that surround the organ (Huvard 1993a), and which act much like a hand squeezing a tube of toothpaste. *Vargula* ostracods are likely able to individually control the different muscles, and their placement around the luminescent organ indicates that each could control a separate type of luminescent compound (Huvard 1993a).

The mucus is thought to envelop the enzyme and substrate to form a distinct bolus, and may be significantly altering the encounter rate of enzyme and substrate, thus controlling the rate of reaction (Huvard 1993a). By preventing the diffusion into the surrounding seawater, it could be increasing the encounter rate of enzyme to substrate, thus increasing the reaction rate. Alternatively, if the mucus is not just surrounding the luminescent molecules but is pervasive throughout the bolus, it could actually slow the diffusion of chemicals to a point that it decreases the reaction rate more than if it were in only seawater. Regardless of its actual effect on the reaction

rate, the lack of variation between pulses during a courtship display indicates that the mucus secretion is as rigidly-controlled as the luminescent compounds.

A question still to be answered is whether there are differences in the concentrations between the molecules released during courtship displays and antipredation behavior. The order of magnitude higher amount of variation in antipredation display decay may be due to a number of factors besides the ostracod itself exhibiting less mechanical control over the secretion. First, a 1st order kinetic reaction rate depends on the volume in which the reaction occurs, with a larger volume corresponding to a slower rate of decay, as the encounter rate of enzyme to substrate would be lower. Indeed, the antipredation episode occupies much larger luminescent volume, especially after being dispersed by the pumping action of the fish's gills. Only by knowing the exact volume in which the antipredation display occurs would we be able to determine whether the majority of the difference between courtship and antipredation display decay rates could be explained by this effect. The presence or absence of mucus in antipredation displays and their probable effect on the diffusion rate of luminescence compounds may also contribute to the differences.

Predator deterrence: Luminescent responses to predation attempts have been shown to deter predators in both terrestrial and marine bioluminescent animals in a variety of ways (Grober 1988; Underwood et al. 1997). The light could function as an aposematic signal warning of distastefulness, it could act as to startle a predator into abandoning a predation attempt, or it could act as a 'flash bulb' effect where predators with highly-sensitive eyes could be effectively blinded, thus allowing the prey to escape (for review see Hastings and Morin 1991; Morin 1983). Our observations of nocturnal predation attempts in the lab show no evidence of the fish predator being blinded, for we have seen them track other ostracods immediately following an antipredation display, therefore making the 'flash bulb' hypothesis

implausible. One would expect that if the fish predator were startled by luminescence that it would immediately expectorate the luminescing ostracod. *Phaeoptyx* cardinalfish often keep a heavily-luminescing *V. annecohenae* in their buccal cavities for several seconds before expectorating them, leading us reject the 'startle' hypothesis as well. Our data, however, show that there is a strong element of distastefulness of these ostracods, indicating that an aposematic function is possible. In all cases but one (which was eaten only after it had apparently exhausted its luminescence stores) live, but not heat-killed, luminescent *V. annecohenae* were expectorated while all other food choices offered were always eaten in both the light and dark. Previous studies have shown that cypridinid luciferase is denatured at high temperatures, while the luciferin is stable (Chase 1950; see Harvey 1952). Therefore, since heat-killed (therefore luciferin-denatured) ostracods are readily eaten, it may be the luciferase or another heat-labile protein that contains the distasteful aspect. Alternatively, by heat killing the ostracod we shut down its behavioral delivery system of distasteful chemicals; thus, the predator may simply not be able to taste the chemical even if it had not been denatured. However, because the nonluminescent *Skogsbergia* ostracods were always eaten, we hypothesize that, barring differences in mechanical defense behavior between the species such as pinching or clawing, whatever causes the unpalatability of the ostracod is from one of the compounds found only in luminescent species.

Regardless of the ultimate source of distastefulness, *V. annecohenae* may actively also be advertising both its unpalatability when participating in courtship luminescence behavior and when releasing an antipredation display. Many luminescent species have been found to be unpalatable (Lloyd 1973; Sydow and Lloyd 1975; Carlson and Copeland 1978; Underwood et al. 1997; De Cock and Mathhysen 1999). Both luminescent pulsing responses to predation as well as pre-predation

glowing signals have been described in lampyrid larvae, and are considered aposematic signals since they both act to deter predators (De Cock and Matthysen 1999, 2003). If luminescence is indeed used as an aposematic signal, then when there *is* luminescent activity, one would expect to see avoidance and decreased predation rates, as has been confirmed on copepods feeding on dinoflagellates (Buskey et al. 1983; Esaias and Curl 1972; Porter and Porter 1979) and toads feeding on firefly larvae (De Cock and Mathhysen 2003). The paucity of observed predation attempts on *V. annecohenae* in the field strongly suggests an avoidance behavior by fishes and other nocturnal predators, even though the obvious signals should make excellent targets for planktivorous fishes. Furthermore, displays are extremely prevalent when conditions permit, and one of us (JGM) has never observed a predation event during courtship over hundreds of hours of night field observations and the other (TJR) has only seen 4 or 5 in nearly 70 hours (Morin 1986; pers. obs.). The effect of depressed predation rates of planktivorous fish during ostracod courtship displays may extend beyond the predator and *V. annecohenae* as well. If nocturnally feeding predators are unable to differentiate between organisms of the same size, they may develop an aversion to all similar-sized prey items during display periods. This selectivity then may reduce predation pressure for other, non-luminescent pelagic species of comparable size. The removal or *V. annecohenae* from shallow reef areas due to anthropogenic reasons (such as light pollution) may therefore cause a dramatic shift in predator-prey dynamics during the periods during which courtship normally occurs.

Although extremely rare in the field, when we *do* see a predation attempt by a fish predator, it is spectacular. A bright blue, fairly long-lasting pulse erupts from the gills of the fish predators **(Figure 4)**, easily drawing the attention of at least human divers. This bright pulse may also attract the predator of the ostracod's attacker as well, which would confirm the presence of the 'burglar alarm' effect put forth by

Burkenroad (1943) and first suggested for ostracods by Morin (1986). Although the hypothesis was conceived over 70 years ago, this phenomenon has only been confirmed to occur much more recently, when copepod grazers on luminescent dinoflagellates were shown to have an increased risk of predation by being 'lit up' for their predators of both fish and squid (Abraham and Townsend 1993; Fleisher and Case 1995; Mensinger and Case 1992). Very preliminary experiments using *V. annecohenae* suggest that a piscivorous predator may orient toward an ostracod antipredation display, but further experiments are warranted. The function of luminescence for predation deterrence via aposematism and the burglar alarm effect may, therefore, offer separate and strong selection pressures for predators to leave the ostracods display unmolested, thus leading to the extreme rarity of observed predation attempts in the field on not only *V. annecohenae*, but all observed ostracods that utilize luminescence in complex courtship displays in the Caribbean.

Conclusion: Although cypridinid ostracod luminescence kinetics are one of the oldest and most heavily-studied luminescent systems, *Vargula annecohenae* is the first species in which the kinetics of two separate behaviors from the field are discussed in any detail. Individual ostracods (male and female, juvenile to adult) are capable of releasing surprising amounts of luminescence into the water column as predator deterrence, and males also exhibit fairly rigid control over the release of luminescence in discrete, complex patterns. The amount of luminescent compound stores a male has readily available may highly influence which mating tactics he uses, therefore potentially highly influencing his mating success. Luminescence may serve three separate roles for male ostracods: 1) for courtship, 2) as an aposematic signal, and 3) as a 'burglar alarm,' where its predator itself is eaten. Although *V. annecohenae* still falls short of the multiple uses for luminescence a flashlight fish exhibits (Morin et al. 1975), its repertoire remains impressive, especially for a tiny, 2mm crustacean.

REFERENCES

Abraham, M.V. and Townsend, L.D. 1993. Bioluminescence in dinoflagellates a test of the burglar alarm hypothesis. *Ecology.* 74: 258-260.

Burkenroad, M.D. 1943. A possible function of bioluminescence. *J. Mar. Res.* 5: 161-164.

Buskey, E., Mills, L., and Swift, E.1983. The effects of dinoflagellate bioluminescence on the swimming behavior of a marine copepod. *Limnol. Oceanogr.*, 28(3):575-579.

Carlson, A.D. and Copeland, J. 1978. Behavioral plasticity in the flash communication systems of fireflies. *Am. Sci.* 66: 340-346.

Chase, A.M. 1950. Studies on cell enzyme systems. 4. The kinetics of heat inactivation of cypridina luciferase. *Jour. Gen. Physiol.* 33 (5): 535-546.

Cohen, A. C. and Morin, J.G. 2003. Sexual morphology, reproduction and the evolution of bioluminescence in Ostracoda. In: *Paleontological Society Papers* [Chapter 4] 9: 37-70.

De Cock, R., and Matthysen, E. 1999. Aposematism and bioluminescence: experimental evidence from glow-worm larvae (Coleoptera: Lampyridae). *Evol. Ecol.* 13: 619-639.

De Cock, R., and Matthysen, E. 2003.Glow-worm larvae bioluminescence (Coleoptera: Lampyridae) operates as an aposematic signal upon toads *(Bufo bufo) Behav. Ecol:* 14 (1): 103- 108.

Esaias, W.E. and Curl, H.C. 1972. Effect of dinoflagellate bioluminescence on copepod ingestion rates. *Limnol. Oceanogr.* 17: 901-906.

Fleisher, K.J. and Case, J.F. 1995. Cephalopod predation facilitated by dinoflagellate luminescence. *Biol. Bull.* 189(3): 263-271.

Gerrish, G.A. and Morin J.G., *in press.*

Grober, M.S. 1988. Brittle-star bioluminescence functions as an aposematic signal to deter crustacean predators. *Anim. Behav. Part 2* 36:493-501.

Haddock, S.H.D., and Case, J.F. 1994. A bioluminescent chaetognath. *Nature* 367 (6460): 225-226.

Haddock, S.H.D., Rivers, T.J., and Robison, B.H. 2001. Can coelenterates make

coelenterazine? Dietary requirements for luminescence in cnidarian bioluminescence. *PNAS* 98 (20): 11148-11151.

Harvey, E.N. 1952. *Bioluminescence*. New York: Academic Press.

Hastings, W.J., and Morin, J.G. 1991. Bioluminescence.Chapter 3 In: Prosser, C.L., ed. *Neural and integrative animal physiology.* Wiley Interscience, New York, pp. 131-170.

Herring, P.J. 1978. *Bioluminescence in action.* New York: Academic Press.

Huvard, A.L. 1993a. Ultrastructure of the light organ and immunocytochemical localization of luciferase in luminescent marine ostracods (Crustacea: Ostracoda: Cypridinidae). *J. Morph.* 218: 181-193.

Huvard, A.L. 1993b. Analysis of visual pigment absorbance and luminescence emission spectra in marine ostrcodes (Crustacea: Ostracoda). *J. Comp. Biochem. Physiol., A.* 104 (2): 333-338.

Jerlov, N.G. 1968. *Optical Oceanography.* New York: Elsevier Pub. Co.

Korniker, L.S. 1984. Cypridinidae of the continental shelves of southwestern North America, the northern Gulf of Mexico, and the West Indies (Ostracoda: Myodocopina). *Smithson. Contrib. Zool.* 309:1-29.

Lloyd, J.E. 1983. Bioluminescent communication in insects. *Annu. Rev. Entom.* 28: 131-160.

Mensinger, A.F. and Case, J.F. 1992. Dinoflagellate luminescence increases susceptibility of zooplankton to teleost predation. *Mar. Biol.* 112: 207-210.

Morin, J.G. 1983. Coastal bioluminescence: patterns and functions. *Bull. Mar. Sci.* 33(4): 787-817.

Morin, J.G. 1986. 'Firefleas' of the sea- luminescent signaling in marine ostracode crustaceans. *Fla. Entom.* 69 (1): 105-121.

Morin, J. G. and Cohen, A.C. 1991. Bioluminescent displays, courtship, and reproduction in ostracodes. In R. Bauer and Martin, J. (Eds.), *Crustacean Sexual Biology:*1-16. New York: Columbia University Press.

Morin, J.G., Harrington, A., Nealson, K., Krieger, N., Baldwin, T.O., and Hastings, J.W. 1975. Light for all reasons- versatility in behavioral repertoire of flashlight fish. *Science.* 190(4209): 74-76.

Porter, K.G. and Porter, J.W. 1979. Bioluminescence in marine plankton: a coevolved antipredation system. *Am. Nat.* 114: 458-461.

Rees J.F., De Wergifosse B., Noiset O., Dubuisson M., Janssens B., and Thompson, E.M. 1998. The origins of marine bioluminescence: Turning oxygen defence mechanisms into deep-sea communication tools. *J. Exp. Bio.* 201 (8): 1211-1221.

Shimomura, O. 2006. *Bioluminescence : chemical principles and methods.* Hackensack, NJ: World Scientific.

Sydow, S.L. and Lloyd, J.E. 1975. Distasteful fireflies sometimes emetic, but not lethal. *Fla. Entom.* 58**:** 312.

Torres and Morin, *in press.*

Tsuji, F. I., Barnes, A.F., and Case, J. F. 1972. Bioluminescence in the marine teleost *Porichthys notatus*, and its induction in a non-luminous form by *Cypridina* (ostracod) luciferin. *Nature* 237: 515-516.

Tsuji, F.I., Lynch, R.V. III, and Haneda, Y. 1970. Studies on the bioluminescence of the marine ostracod crustacean *Cypridina serrata. Biol. Bull.* 139: 386-401.

Underwood, T.J., Tallamy, D.W. and Pesek, J.D. 1997. Bioluminescence in firefly larvae: a test of the aposmatic display hypothesis (Coleoptera: Lampyridae). *J. Insect Behav.*, 10: 365-370.